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(54) Title: NOVEL COMPOUNDS AND COMPOSITIONS FOR TREATING HEPATITIS C INFECTIONS

(57) Abstract

The present invention relates to novel biheterocyclic derivatives which are serine protease inhibitors; the pharmaceutically acceptable salts and *N*–oxides thereof; their uses as therapeutic agents and the methods of their making.

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NOVEL COMPOUNDS AND COMPOSITIONS FOR TREATING HEPATITIS C INFECTIONS

This application claims the benefit under 35 U. S. C. 119(e)(1) of prior filed provisional application 60/103,085 filed October 5, 1998.

Field of the Invention:

The present invention relates to a novel class of compounds which are effective in inhibiting the activity of serine proteases, particularly the hepatitis C virus protease NS3, and in treating hepatitis C viral infections. The present invention also relates to methods for using the compounds in treating hepatitis C viral infections and methods for making and pharmaceutical compositions containing the compounds.

Description of the Field:

Viral hepatitis is a hepatocellular inflammatory disease caused by specific hepatotrophic viruses. The disease can range from acute hepatitis progressing to chronic persistent hepatitis and eventual cirrhosis. Parenterally transmitted non-A, non-B viral infections cause 90 to 95% of all transfusion-associated viral hepatitis and may account for as many as 300,000 cases of hepatitis per year in the United States. Hepatitis C virus (HCV) is the apparent causative agent for most non-A, non-B hepatitis infections and is most likely the leading cause of chronic liver disease in the Western world.

The HCV genome encodes for a single polypeptide having approximately 3010 amino acids. Five nonstructural regions, NS1 to NS5, are encoded toward the 3'-end of the genome and several structural proteins are encoded near the 3'-end of the genome. The NS3 region encodes for a serine protease that, along with an associated cofactor NS4A, is involved in processing the HCV translation product into its individual functioning structural and nonstructural proteins. Hence, functional NS3 protease is a necessary component of HCV replication.

Patients with acute HCV hepatitis may recover without medical intervention.

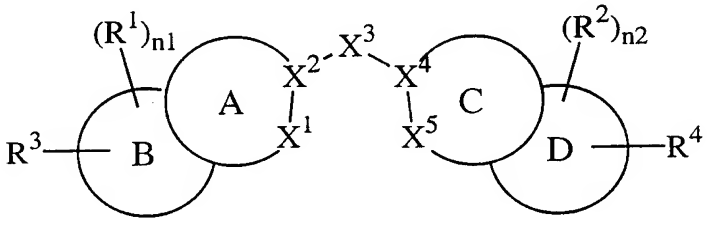
However, about half of all acute infections progress to chronic persistent hepatitis, which left untreated can lead to cirrhosis and eventual death. The hepatocellular inflammatory effects of the chronic HCV hepatitis can be ameliorated with corticosteroid treatments. Antiviral agents such as acyclovir or interferon-α are used to treat HCV infection. Interferon-α, the only approved anti-HCV therapeutic agent, is expensive and must be administered by subcutaneous injection three times a week for up to six months. Interferon-α produces improvements in liver enzymes and histology; however, HCV RNA titer frequently remains high despite long term chemotherapy. Moreover, a significant population of patients relapse when drug therapy is stopped. The overall success in treating HCV hepatitis with interferon-α is about 25%.

Agents which inhibit the processing of viral protein can be effective anti-viral agents. Hence, the NS3 serine protease is a rational target for designing new and effective anti-HCV chemotherapies. Peptide-like NS3 protease inhibitors are known and described in PCT International Applications WO 98/17679 and WO 98/22496 as anti-HCV chemotherapeutic agents. The discovery and development of low molecular weight, non-peptide inhibitors of the NS3 serine protease will provide a highly effective means for treating HCV infections.

The disclosures of documents, including patents and patent applications, referred to throughout this application are incorporated herein by reference.

SUMMARY OF THE INVENTION

This application relates to a compound of Formula I:



I

in which:

n1 is 0, 1, 2, 3 or 4;

n2 is 0, 1, 2 or 3;

A together with B comprise a fused heterobicyclic radical containing 8 to 12 annular atoms, wherein each ring contains 5 to 7 annular members, each annular atom optionally is a heteroatom moiety, X^1 and X^2 are adjacent annular members of an aromatic ring and X^1 is a heteroatom moiety selected from –N=, $–NR^5–$, –O– and –S–, wherein R^5 is hydrogen or (C_{1-6})alkyl;

C together with D comprise a fused heterobicyclic radical containing 8 to 12 annular atoms, wherein each ring contains 5 to 7 annular members, each annular atom optionally is a heteroatom, X^4 and X^5 are adjacent annular members of an aromatic ring and X^5 is a heteroatom moiety selected from –N=, $–NR^6–$, –O– and –S–, wherein R^6 is hydrogen or (C_{1-8})alkyl optionally substituted with one to two substituents independently selected from halo, tri(C_{1-6})alkylammonio, $–NR^7R^7$, $–C(O)NR^7R^7$, $–OR^7$, $–C(O)OR^7$, $–OC(O)R^7$ or $–S(O)_2OR^7$, wherein R^7 at each occurrence independently is hydrogen or (C_{1-6})alkyl;

X^3 is –O–, –S–, –S(O)–, $–S(O)_2–$, –C(O)–, $–NR^8–$ or $–CR^8R^9–$, wherein R^8 is hydrogen, halo, (C_{1-6})alkyl or together with R^9 forms (C_{2-6})alkylene or (C_{1-6})alkylidene and R^9 is hydrogen, halo, (C_{1-6})alkyl or as defined above, wherein any 1 to 3 carbon atoms with a free valence comprising R^8 and/or R^9 optionally independently are substituted with halo, tri(C_{1-6})alkylammonio, $–NR^{10}R^{10}$, $–C(O)NR^{10}R^{10}$, $–OR^{10}$, $–C(O)OR^{10}$ or $–OC(O)R^{10}$, wherein R^{10} at each occurrence independently is hydrogen or (C_{1-6})alkyl;

R^1 at each occurrence independently is (C_{1-6})alkyl, (C_{1-6})alkyloxy, (C_{1-6})alkanoyloxy, (C_{1-6})alkylthio, halo, hydroxy or mercapto and bonded to any annular carbon atom with a free valence comprising B;

R^2 at each occurrence independently is (C_{1-6})alkyl, (C_{1-6})alkyloxy, (C_{1-6})alkanoyloxy, (C_{1-6})alkylthio, halo, hydroxy or mercapto and bonded to any annular carbon atom with a free valence comprising C;

R^3 is cyano, $–R^{11}$, $–CR^{12}R^{12}NR^{11}R^{13}$, $–C(NR^{13})R^{11}$, $–C(O)R^{11}$, $–C(NR^{13})NR^{11}R^{13}$, $–C(O)NR^{11}R^{13}$, $–C(O)OR^{11}$, $–S(O)R^{11}$, $–S(O)_2R^{11}$, $–S(O)_2NR^{11}R^{13}$ or $–S(O)_2OR^{11}$ and bonded to

any annular atom with a free valence comprising B, wherein:

R^{11} is hydrogen, (C_{1-6})alkyl, cyclo(C_{3-6})alkyl(C_{0-3})alkyl, heterocyclo(C_{3-6})alkyl(C_{0-3})alkyl, (C_{6-10})aryl(C_{0-3})alkyl, hetero(C_{5-14})aryl(C_{0-3})alkyl, polycyclo(C_{9-10})aryl(C_{0-3})alkyl or heteropolycyclo(C_{8-10})aryl(C_{0-3})alkyl; wherein any alkyl moiety comprising R^{11} optionally independently is substituted with 1 to 3 substitutents selected from $-P(O)(OR^{14})OR^{14}$, $-S(O)_2OR^{14}$ and $-C(O)OR^{14}$ and any 1 to 3 annular carbon atoms with free valences of any aromatic ring comprising R^{11} optionally independently are substituted with halo, nitro, cyano, optionally halo-substituted (C_{1-6})alkyl, $-OR^{14}$, $-C(O)OR^{14}$, $-C(O)NR^{14}R^{14}$, $-X^6NR^{14}R^{14}$, $-X^6NR^{14}C(O)NR^{14}R^{14}$ or $-X^6NR^{14}C(NR^{14})NR^{14}R^{14}$, wherein X^6 is a bond or methylene and R^{14} at each occurrence independently is hydrogen or (C_{1-6})alkyl,

R^{12} at each occurrence independently is hydrogen, (C_{1-3})alkyl or together with another R^{12} and the carbon atom to which both are attached forms cyclopropyl and

R^{13} at each occurrence independently is hydrogen or (C_{1-6})alkyl; and

R^4 is $-R^{15}$, $-OR^{15}$, $-NR^{15}R^{16}$, $-SR^{15}$, $-S(O)R^{15}$, $-S(O)_2R^{15}$, $-S(O)_2OR^{15}$, $-S(O)_2NR^{15}R^{16}$, $-N(R^{16})S(O)_2R^{15}$, $-C(O)R^{15}$, $-C(O)OR^{15}$, $-C(O)NR^{15}R^{16}$, $-N(R^{16})C(O)R^{15}$, $-OC(O)NR^{15}R^{16}$, $-N(R^{16})C(O)OR^{15}$ or $-N(R^{16})C(O)NR^{15}R^{16}$, and bonded to any annular carbon atom with a free valence comprising C, wherein:

R^{15} is (C_{1-6})alkyl substituted with 1 to 2 radicals selected from $-P(O)(OR^{17})OR^{17}$ and $-S(O)_2OR^{17}$ and optionally substituted with 1 to 2 radicals $-C(O)OR^{17}$ groups, wherein R^{17} is hydrogen or (C_{1-6})alkyl, and

R^{16} is hydrogen or (C_{1-6})alkyl; and the *N*-oxide derivatives, prodrug derivatives, protected derivatives, individual isomers, mixtures of isomers and pharmaceutically acceptable salts thereof.

A second aspect of this invention is a pharmaceutical composition which contains a compound of the invention or a *N*-oxide derivative, prodrug derivatives, individual isomer, mixture of isomers or pharmaceutically acceptable salt thereof in admixture with one or more suitable excipients.

A third aspect of this invention is a method of treating a patient infected with hepatitis

C virus, which method comprises administering to the patient a therapeutically effective amount of a compound of the invention or a *N*-oxide derivative, prodrug derivative, individual isomer, mixture of isomers or pharmaceutically acceptable salt thereof.

A fourth aspect of this invention is the processes for preparing compounds of the invention and the *N*-oxide derivatives, prodrug derivatives, protected derivatives, individual isomers, mixtures of isomers and pharmaceutically acceptable salts thereof as set forth in "Detailed Description of the Invention".

DETAILED DESCRIPTION OF THE INVENTION

Definitions:

Unless otherwise stated, the following terms used in the specification and claims are defined for the purposes of this application and have the meanings given below:

"Alkanoyl" means the radical –C(O)R, wherein R is alkyl as defined in the Detailed Description of the Invention, having overall the number of carbon atoms indicated (e.g., (C_{1-6})alkanoyl includes the radicals formyl, acetyl, propionyl, butyryl, isobutyryl, crotonoyl, isocrotonyl, etc.).

"Alkyl", for the purposes of this application, means a straight or branched, saturated or unsaturated aliphatic hydrocarbon radical having the number of carbon atoms indicated, and any ketone, thioketone or iminoketone thereof (e.g., (C_{1-8})alkyl includes methyl, ethyl, propyl, isopropyl, butyl, *sec*-butyl, isobutyl, *tert*-butyl, vinyl, allyl, 1-propenyl, isopropenyl, 1-butenyl, 2-butenyl, 3-butenyl, 2-methylallyl, ethynyl, 1-propynyl, 2-propynyl, 3-oxopentyl, 3-thioxopentyl, 3-iminopentyl, etc.). The term "(C_0)alkyl", as in (C_{6-10})aryl(C_{0-3})alkyl, means that the linking alkyl moiety does not exist and the aryl group is bonded directly to the point of attachment as a subsitutent.

"Alkylene" means a saturated or unsaturated hydrocarbon divalent radical having the number of carbon atoms indicated and any ketone, thioketone, iminoketone derivative thereof (e.g., (C_{2-6})alkylene includes methylene (–CH_2–), ethylene (–CH_2CH_2–), methylethylene,

vinylene, ethynylene, trimethylene ($-CH_2CH_2CH_2-$), 2-oxotrimethylene ($-CH_2C(O)CH_2-$), 2-thiatrimethylene ($-CH_2C(S)CH_2-$), 2-iminotrimethylene ($-CH_2C(NH)CH_2-$), propenylene ($-CH_2CH=CH-$ or $-CH=CHCH_2-$), propanylylidene ($=CHCH_2CH_2-$), propendiylene ($=CHCH=CH-$), tetramethylene, pentamethylene, etc.).

"Alkylidene" means the radical $=CRR$, wherein each R independently is hydrogen or alkyl, as defined in the Detailed Description of the Invention, having overall the number of carbon atoms indicated (e.g., (C_{1-6})alkylidene includes methylidene, ethylidene, propylidene, isopropylidene, etc.).

"Alkyloxy" means the radical $-OR$, wherein R is alkyl as defined in the Detailed Description of the Invention, having the number of carbon atoms indicated (e.g., (C_{1-6})alkyloxy includes the radicals methoxy, ethoxy, propoxy, isopropoxy, butoxy, *sec*-butoxy, isobutoxy, *tert*-butoxy, vinyloxy, allyloxy, 1-propenyloxy, isopropenyloxy, 1-butenyloxy, 2-butenyloxy, 3-butenyloxy, 2-methylallyloxy, ethynyloxy, 1-propynyloxy, 2-propynyloxy, etc.).

"Ammonio" means the radical $-NH_3^+$.

"Amino" means the radical $-NH_2$.

"Aryl" means an aromatic monocyclic or fused polycyclic hydrocarbon radical containing the number of carbon atoms indicated, wherein each ring contained therein is comprised of 6 annular members (e.g., (C_{6-14})aryl includes phenyl, naphthyl, anthracenyl, phenanthrenyl, etc.).

"Carbamoyl" means the radical $-C(O)NH_2$.

"Carboxy" means the radical $-C(O)OH$.

"Cyano" means the radical $-CN$.

"Cycloalkyl" means a saturated or unsaturated, monocyclic or fused polycyclic hydrocarbon radical containing the number of carbon atoms indicated, wherein each ring contained therein is comprised of 3 to 8 annular members, and any carbocyclic ketone, thioketone and iminoketone derivative thereof. For example, (C_{3-14})cycloalkyl includes cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cyclohexenyl, 2,5-cyclohexadienyl, bicyclo[2.2.2]octyl, oxocyclohexyl, dioxocyclohexyl, thiocyclohexyl, and the like.

"Deprotecting" refers to removing any protective groups present after the selective reaction has been carried out.

"Fused heterobicyclic radical" means a heterocyclic radical containing two fused rings having the number of annular members indicated, wherein at least two annular members of one ring are common to the second ring, and the carbocyclic ketone and thioketone derivatives thereof. For examplea heterobicyclic radical containing from 8 to 12 annular atoms includes 1*H*-benzimidazol-2-yl, 1*H*-naphtho[2,3-*d*]imidazol-2-yl, 1*H*-imidazo[4,5-*f*]quinolin-2-yl, 1*H*-imidazo[4,5-*b*]pyridin-2-yl, 2,6-dioxo-2,3,6,7-tetrahydro-1*H*-purin-8-yl, 2,6-dithioxo-2,3,6,9-tetrahydro-1*H*-purin-8-yl, 7*H*-purin-8-yl, 1,6-dihydrocyclopentaimidazol-2-yl, 4-quinolin-2-yl, and the like.

"Free valence", when referring to atoms in the compounds of the invention, means that the atom(s) referred has the capacity to form a bond with another molecule, other than hydrogen, and, thus, comprise a substituted atom. For the purposes of the this application, when referring to a compound of the invention by formula and the attachement of a free valence not designated, it is to be understood that the reference is to all attachments possible, including to hydrogen, optional bonds or optional substituents. Hence, for example, the compound of Formula II, infra., in which the annullar atom X^8 is N, refers to instances wherein the indicated nitrogen atom is attached to an annular carbon atom (i.e., when the optional bond is present) and a hydrogen atom (i.e., when the optional bond is absent).

"Halo" means fluoro, chloro, bromo or iodo.

"Heteroatom moiety", unless indicated otherwise, means a moiety selected from –N=, $-NR^{18}-$, –O–, –S–, –S(O)–, $-S(O)_2-$ and $-P(O)(OR^{18})-$, wherein R^{18} is hydrogen or (C_{1-6})alkyl.

"Heteroaryl" means an aromatic monocyclic or fused polycyclic divalent radical having the number of annular atoms indicated, wherein each ring contained therein is comprised of 5 to 6 annular members and one or more of the annular atoms is a heteroatom moiety, as defined in the Detailed Description of the Invention, and each ring contained therein is comprised of 5 to 6 annular members (e.g., hetero(C_{5-14})aryl includes thienyl, furyl, pyrrolyl, pyrimidinyl, isoxazolyl, oxaxolyl, indolyl, benzo[*b*]thienyl, isobenzofuranyl, purinyl, isoquinolyl, pterdinyl, perimidinyl, imidazolyl, pyridyl, pyrazolyl, pyrazinyl, quinolyl, etc.).

"Heterocycloalkyl" means cycloalkyl, as defined above, except one or more of the annular carbon atoms indicated are replaced by a heteroatom moiety, as defined in the Detailed Description of the Invention, and any carbocyclic ketone, thioketone or iminoketone derivative thereof. For example, the term heterocyclo(C_{5-14})alkyl includes piperidyl, pyrrolidinyl, pyrrolinyl, imidazolidinyl, quinuclidinyl, morpholinyl, and the like.

"Heterocycloalkylene" means cycloalkylene, as defined above, except one or more of the annular carbon atoms indicated is replaced by a heteroatom moiety, as defined in the Detailed Description of the Invention, and any carbocyclic ketone, thioketone or iminoketone derivative thereof. For example, the term heterocyclo(C_{3-14})alkylene includes piperidylene, pyrrolidinylene, pyrrolinylene, imidazolidinylene, quinuclidinylene, morpholinylene, and the like.

"Heteropolycycloaryl" means polycycloaryl, as defined below, except one or more of the annular carbon atoms indicated are replaced by a heteroatom moiety, as set defined in the Detailed Description of the Invention, and any carbocyclic ketone, thioketone or iminoketone derivative thereof. For example, heteropolycyclo(C_{8-10})alkyl includes 3,4-dihydro-2*H*-quinolinyl, 5,6,7,8-tetrahydroquinolinyl, 3,4-dihydro-2*H*-[1,8]naphthyridinyl, 2,4-dioxo-3,4-dihydro-2*H*-quinazolinyl, 3-oxo-2,3-dihydrobenzo[1,4]oxazinyl, and the like.

"Hydroxy" means the radical –OH.

"Iminoketone" means the derivative –C(NR)–, wherein R is hydrogen or alkyl as defined in the Detailed Description of the Invention.

"Isomers" mean compounds of the invention having identical molecular formulae but differ in the nature or sequence of bonding of their atoms or in the arrangement of their atoms in space. Isomers that differ in the arrangement of their atoms in space are termed "stereoisomers". Stereoisomers that are not mirror images of one another are termed "diastereomers" and stereoisomers that are nonsuperimposable mirror images are termed "enantiomers" or sometimes "optical isomers". A carbon atom bonded to four nonidentical substituents is termed a "chiral center". A compound with one chiral center has two enantiomeric forms of opposite chirality is termed a "racemic mixture". A compound that has more than one chiral center has 2^{n-1} enantiomeric pairs, where n is the number of chiral

centers. Compounds with more than one chiral center may exist as either an individual diasteromer or as a mixture of diastereomers, termed a "diastereomeric mixture". When one chiral center is present a stereoisomer may be characterized by the absolute configuration of that chiral center. Absolute configuration refers to the arrangement in space of the substituents attached to the chiral center. The substituents attached to the chiral center under consideration are ranked in accordance with the *Sequence Rule* of Cahn, Ingold and Prelog and the absolute descriptor *R* or *S* is cited in parenthesis followed by a hyphen and the chemical name of the compound. Compounds of the invention that contain a chiral center can exist as individual stereoisomers or mixtures of stereoisomers. For the purposes of the this application when referring to a compound of the invention by name or by formula and the configuration is not designated, it is to be understood that the reference is to all possible configurations of the compound and the mixtures, racemic or otherwise, thereof.

"Ketone" means the derivative –C(O)–.

"Leaving group" has the meaning conventionally associated with it in synthetic organic chemistry, i.e., an atom or group displaceable under alkylating conditions, and includes, halogen, hydroxy, alkyloxy, alkylsulfonloxy (e.g., mesyloxy, ethanesulfonyloxy, etc.), arylsulfonyloxy (e.g., benzenesulfonyloxy and tosyloxy, thienyloxy), dihalophosphinoyloxy, tetrahalophosphaoxy, and the like.

"Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where the event or circumstance occurs and instances in which it does not. For example, the phrase "optionally independently are substituted with" means that the group referred to may or may not be substituted in order to fall within the scope of the invention.

"*N*-oxide derivatives" means derivatives of compounds of the invention in which nitrogens are in an oxidized state (i.e., O←N) and which possess the desired pharmacological activity. The *N*-oxide derivatives of compounds of the invention can be prepared by methods known to those of ordinary skill in the art.

"Pharmaceutically acceptable" means that which is useful in preparing a pharmaceutical composition that is generally safe, non-toxic and neither biologically nor

otherwise undesirable and includes that which is acceptable for veterinary use as well as human pharmaceutical use.

"Pharmaceutically acceptable salts" means salts of compounds of the invention which are pharmaceutically acceptable, as defined above, and which possess the desired pharmacological activity. Such salts include acid addition salts formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like; or with organic acids such as acetic acid, propionic acid, hexanoic acid, heptanoic acid, cyclopentanepropionic acid, glycolic acid, pyruvic acid, lactic acid, malonic acid, succinic acid, malic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, *o*-(4-hydroxybenzoyl)benzoic acid, cinnamic acid, madelic acid, methanesulfonic acid, ethanesulfonic acid, 1,2-ethanedisulfonic acid, 2-hydroxyethanesulfonic acid, benzenesulfonic acid, *p*-chlorobenzenesulfonic acid, 2-naphthalenesulfonic acid, *p*-toluenesulfonic acid, camphorsulfonic acid, 4-methylbicyclo[2.2.2]oct-2-ene-1-carboxylic acid, glucoheptonic acid, 4,4'-methylenebis(3-hydroxy-2-ene-1-carboxylic acid), 3-phenylpropionic acid, trimethylacetic acid, tertiary butylacetic acid, lauryl sulfuric acid, gluconic acid, glutamic acid, hydroxynaphthoic acid, salicylic acid, stearic acid, muconic acid and the like.

Pharmaceutically acceptable salts also include base addition salts which may be formed when acidic protons present are capable of reacting with inorganic or organic bases. Acceptable inorganic bases include sodium hydroxide, sodium carbonate, potassium hydroxide, aluminum hydroxide and calcium hydroxide. Acceptable organic bases include ethanolamine, diethanolamine, triethanolamine, tromethamine, *N*-methylglucamine and the like.

"Phosphono" means the radical $-P(O)(OH)_2$.

"Polycycloaryl" means a fused polycyclic radical containing the number of carbon atoms indicated, wherein at least one, but not all, of the fused rings comprising the radical is aromatic and each ring contained therein is comprised of five to six annular members, and any carbocyclic ketone and thioketone derivative thereof. For example, polycyclo(C_{9-10})aryl includes indanyl, indenyl, 1,2,3,4-tetrahydronaphthyl, 1,2-dihydronaphthyl,

2,4-dioxo-1,2,3,4-tetrahydronaphthyl, and the like.

"Prodrug derivatives" means derivatives of compounds of the invention which are converted *in vivo* to the corresponding non-derivatized form of a compound of the invention. Suitable prodrug derivatives include those compounds of the invention in which one or more nitrogen and/or oxygen atoms with a free valence are substituted with a group which is readily cleavable by *in vivo* processes. For example, prodrug derivatives of compounds of the invention may contain one or more *N*-substituted amino groups (e.g., $-NH_2(R^{19})$), *N*-substituted nitrogen atoms incorporated into an aliphatic, alicyclic or aromatic structure (e.g., $-N(R^{19})-$), *N*-substituted imino or amidino groups (e.g., $-C(NR^{19})H$, $-C(NR^{19})NH_2$ or $-C(NH)NHR^{19}$), *N*-substituted guanidino groups (e.g., $-NHC(NR^{19})NHR^{19}$, $-NHC(NH)NHR^{19}$ or $-NHC(NR^{19})NH_2$), and the like, in which R^{19} is (i) $-C(O)R^{20}$ or $-CH(R^{21})OC(O)R^{20}$, wherein R^{20} is (C_{1-10})alkyl, (C_{1-10})alkyloxy, carbamoyl, (C_{1-10})alkylcarbamoyl, di(C_{1-10})alkylcarbamoyl, *cis*-2-(C_{1-10})alkanoyloxyphenylvinyl, 3-(C_{1-10})alkanoyloxybutyryl, (C_{3-10})cycloalkyl, hetero(C_{3-10})cycloalkyl, (C_{6-10})aryl or hetero(C_{5-10})aryl and R^{21} is hydrogen or (C_{1-10})alkyl; (ii) $-X^7-R^{22}$, wherein X^7 is (C_{1-10})alkylene and R^{22} is carboxy; or (iii) $-C(O)OCH(R^{23})OC(O)R^{24}$, wherein R^{23} is hydrogen, (C_{1-10})alkyl or (C_{3-10})cycloalkyl and R^{24} is (C_{1-10})alkyl or (C_{3-10})cycloalkyl. In addition, prodrug derivatives of compounds of the invention may contain one or more *N*-hydroxylated imino or amidino groups (e.g., $-C(NOR^{25})H$, $-C(NOR^{25})NH_2$ or $-C(NH)NHOR^{25}$) or *N*-hydroxylated guanidino groups (e.g., $-NHC(NOR^{25})NH_2$, $-NHC(NH)NHOR^{25}$), in which R^{25} is hydrogen, methyl, $-C(O)R^{26}$ or $-CH(R^{27})OC(O)R^{26}$, wherein R^{26} is (C_{1-10})alkyl or (C_{3-10})cycloalkyl and R^{27} is hydrogen or (C_{1-10})alkyl; *N*-substituted hydroxy groups (e.g.,$-OR^{28}$), in which R^{28} is $-C(O)R^{19}$ or $-CH(R^{20})OC(O)R^{19}$, wherein R^{19} and R^{20} are as defined above; and/or ester derivatives of carboxylic acids (e.g., $-C(O)OR^{29}$), phosphonic acids (e.g., $-P(O)(OR^{29})$) and sulfonic acids (e.g., $-S(O)_2OR^{29}$ wherein R^{29} is (C_{1-10})alkyl, (C_{3-10})cycloalkyl or $-C(O)OCH(R^{23})OC(O)R^{24}$, wherein R^{23} and R^{24} are as defined above.

"Protective group" has the meaning conventionally associated with it in synthetic organic chemistry, i.e., a group which selectively blocks one reactive site in a multifunctional compound such that a chemical reaction can be carried out selectively at another unprotected

reactive site and which can be readily removed after the selective reaction is completed.

"Protected derivatives" means derivatives of compounds of the invention in which a reactive site or sites are blocked with protective groups. Protected derivatives of compounds of the invention are useful in the preparation of compounds of the invention. Suitable protective groups for reactive nitrogen atoms include *tert*-butoxycarbonyl, benzyloxycarbonyl and any other suitable amino protective groups (e.g., see T.W. Greene, *Protective Groups in Organic Synthesis*, John Wiley & Sons, Inc. 1981).

"Therapeutically effective amount" means that amount which, when administered to a patient is effective for treating a disease.

"Thioketone" means the derivative –C(S)–.

"Treatment" or "treating" refers to any administration of a compound of the present invention and includes:

(1) preventing the disease from occurring in a patient which may be predisposed to the disease but does not yet experience or display the pathology or symptoms of the disease,

(2) inhibiting the disease, i.e., arresting development of its pathology and/or symptoms, or

(3) ameliorate the disease, i.e., reversing its pathology and/or symptoms.

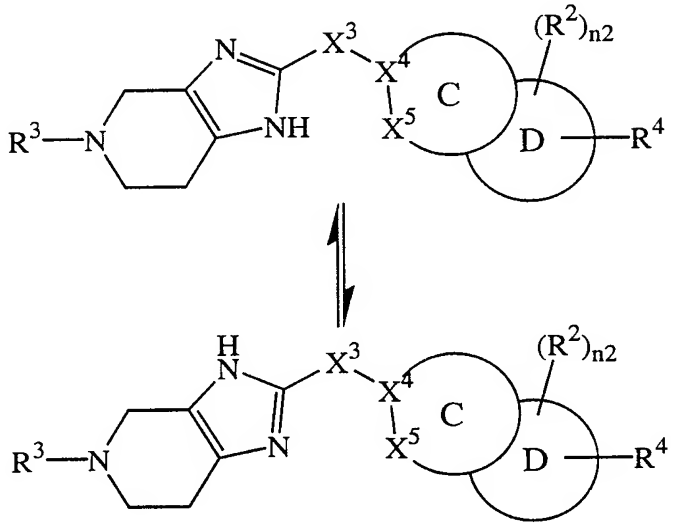
"Sulfo" means the radical –S(O)OH.

The compounds of the invention and the intermediates and starting materials are named by AUTONOM Version 2.0 by Beilstein-Institut and Springer-Verlag Berlin Heidelberg, a fully automatic computerized system for assigning IUPAC systematic nomenclature directly from the structural diagrams of organic compounds.. For example, a compound of Formula I in which:

A together with B comprise 1*H*-benzoimidazol-2-yl, C together with B comprise 6-(1-carboxy-2-phosphonoethylcarbamoyl)-1-methyl-1*H*-benzoimidazol-2-yl and X^3 is $-CH_2(CH_3)-$ is named 2-({2-[1-(1*H*-benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid.

Certain compounds of the invention exist in tautomeric equilibrium. For example,

compounds of Formula I in which A together with B comprise 4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridin-2-yl exist in equilibrium between tautomers of the following formulae:



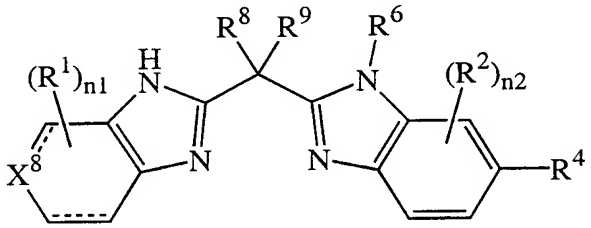
and, hence, while the compounds of this invention may be named, illustrated or otherwise described in this application as one possible tautomer, it is to be understood that all possible tautomers are meant to be encompassed by such names, illustrations and descriptions. Thus, the name 2-({2-[1-(5-hexylcarbamoyl-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridin-2-yl)ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid is meant to include its tautomer 2-({2-[1-(6-hexylcarbamoyl-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridin-2-yl)ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid.

Presently Preferred Embodiments:

While the broadest definition of this Invention is set forth in the Summary of the Invention, certain aspects of the Invention are preferred. A preferred aspect of the Invention is a compound of Formula I in which A together with B and C together with D comprise fused heterobicyclic radicals wherein A and C each contain 5 annular members and B and D

each contain 6 annular members and X^1 and X^2 and X^4 and X^5 are adjacent members of an oxazol-2-yl, 1*H*-imidazol-2-yl or thiazol-2-yl ring.

A preferred aspect of the Invention are compounds of Formula II:



II

in which:

the dashed lines independently represent optional bonds;

n1 is 0, 1, 2, 3 or 4;

n2 is 0, 1, 2 or 3;

X^8 is C, N, CR^3 or NR^3, wherein R^3 is cyano, (C_{1-6})alkyl, $-C(O)R^{11}$, $-C(O)NR^{11}R^{13}$ or $-C(O)OR^{11}$, wherein R^{11} independently is hydrogen, (C_{1-6})alkyl or (C_{1-4})aryl(C_{0-4})alkyl, R^{13} is hydrogen or (C_{1-6})alkyl and any alkyl moiety comprising R^{11} optionally independently is substituted with 1 to 3 substitutents selected from $-P(O)(OR^{14})OR^{14}$, $-S(O)_2OR^{14}$ and $-C(O)OR^{14}$, wherein R^{14} at each occurrence independently is hydrogen or (C_{1-6})alkyl; provided that when X^8 is NR^3 the adjacent optional bond is not present and, unless indicated otherwise, any free valence of an annular atom is occupied by a hydrogen atom;

R^1 and R^2 at each occurrence independently are (C_{1-6})alkyl, (C_{1-6})alkyloxy, halo or hydroxy and bonded to any annular carbon atom with a free valence;

R^4 is $-C(O)NR^{15}R^{16}$, wherein:

R^{15} is (C_{1-6})alkyl substituted with 1 to 2 radicals selected from $-P(O)(OR^{17})OR^{17}$ and $-S(O)_2OR^{17}$ and optionally substituted with 1 to 2 $-C(O)OR^{17}$ groups, wherein R^{17} is hydrogen or (C_{1-6})alkyl, and

R^{16} is hydrogen or (C_{1-6})alkyl;

R^6 is (C_{1-6})alkyl optionally substituted with one to two substituents independently selected from halo, tri(C_{1-6})alkylammonio, $-NR^7R^7$, $-C(O)NR^7R^7$, $-OR^7$, $-C(O)OR^7$,

$-OC(O)R^7$ or $-S(O)_2OR^7$, wherein R^7 at each occurrence independently is hydrogen or (C_{1-6})alkyl; and

R^8 and R^9 independently are hydrogen, halo or (C_{1-6})alkyl, wherein any 1 to 3 carbon atoms with a free valence comprising R^8 and/or R^9 optionally independently are substituted with halo, tri(C_{1-6})alkylammonio, $-NR^{10}R^{10}$, $-C(O)NR^{10}R^{10}$, $-OR^{10}$, $-C(O)OR^{10}$ or $-OC(O)R^{10}$, wherein R^{10} at each occurrence independently is hydrogen or (C_{1-6})alkyl.

A preferred aspect of the invention are compounds of Formula II in which both of the optional bonds are present, n1 and n2 each are 0, X^8 is N or CR^3, R^6 is (C_{1-4})alkyl, R^8 is hydrogen or methyl and R^9 is hydrogen; preferably wherein R^3 is acetyl, benzyloxycarbonyl, cyano or $-C(O)NR^{11}R^{13}$, wherein R^{11} and R^{13} independently are hydrogen or methyl.

A preferred aspect of the invention are compounds of Formula II in which neither of the optional bonds are present, n1 and n2 are 0, X^8 is NR^3, R^6 is (C_{1-4})alkyl, R^8 is hydrogen or methyl and R^9 is hydrogen; preferably wherein R^3 is acetyl, benzyloxycarbonyl or $-C(O)NR^{11}R^{13}$, wherein R^{11} and R^{13} independently are hydrogen or methyl.

A preferred aspect of the invention are compounds of Formula II in which both of the optional bonds are present, n1 is 0, 1, 2, 3 or 4; n2 is 0; X^8 is C; R^1 at each occurrence is chloro, fluoro or hydroxy; R^6 is (C_{1-4})alkyl; R^8 is hydrogen or methyl; and R^9 is hydrogen.

Pharmacology and Utility:

The compounds of the invention are serine protease inhibitors and/or are intermediates useful in the preparation of the compounds of the invention. In particular, the compounds inhibit HCV protease NS-3 and, as such, are useful in treating HCV infections. Methods for testing the compounds of the invention for their serine protease inhibitory activity are known in the art. Typically, these methods measure the enzyme-induced hydrolysis of peptide-based fluorogenic substances. Details of an exemplary procedure for measuring NS3 inhibitory activity are described in Example 25, infra.

The compounds of the invention may be administered alone to treat patients with HCV infections or in combination with other anti-viral agents such as α-, β- or γ-interferons, ribavirin, amantadine and the like.

Compositions and Administration:

In general, compounds of the invention will be administered in therapeutically effective amounts via any of the usual and acceptable modes known in the art, either singly or in combination with another therapeutic agent. A therapeutically effective amount may vary widely depending on the severity of the disease, the age and relative health of the subject, the potency of the compound used and other factors. For example, therapeutically effective amounts of a compound of the invention may range from 1 microgram per kilogram body weight (μg/kg) per day to 10 milligram per kilogram body weight (mg/kg) per day, typically 10 μg/kg/day to 1 mg/kg/day. Therefore, a therapeutically effective amount for a 80 kg human patient may range from 80 μg/day to 100 mg/day, typically 0.1 mg/day to 10 mg/day. In general, one of ordinary skill in the art, acting in reliance upon personal knowledge and the disclosure of this Application, will be able to ascertain a therapeutically effective amount of a compound of the invention for treating a given patient.

The compounds of the invention can be administered as pharmaceutical compositions by one of the following routes: oral, systemic (e.g., transdermal, intranasal or by suppository) or parenteral (e.g., intramuscular, intravenous or subcutaneous). Compositions can take the form of tablets, pills, capsules, semisolids, powders, sustained release formulations, solutions, suspensions, elixirs, aerosols, or any other appropriate composition and are comprised of, in general, a compound of the invention in combination with at least one pharmaceutically acceptable excipient. Acceptable excipients are non-toxic, aid administration, and do not adversely affect the therapeutic benefit of the active ingredient. Such excipient may be any solid, liquid, semisolid or, in the case of an aerosol composition, gaseous excipient that is generally available to one of skill in the art.

Solid pharmaceutical excipients include starch, cellulose, talc, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, and the like. Liquid and semisolid excipients may be selected from water, ethanol, glycerol, propylene glycol and various oils, including those of petroleum, animal, vegetable or synthetic origin (e.g., peanut oil, soybean oil, mineral oil, sesame oil, etc.). Preferred liquid carriers, particularly for injectable

solutions, include water, saline, aqueous dextrose and glycols.

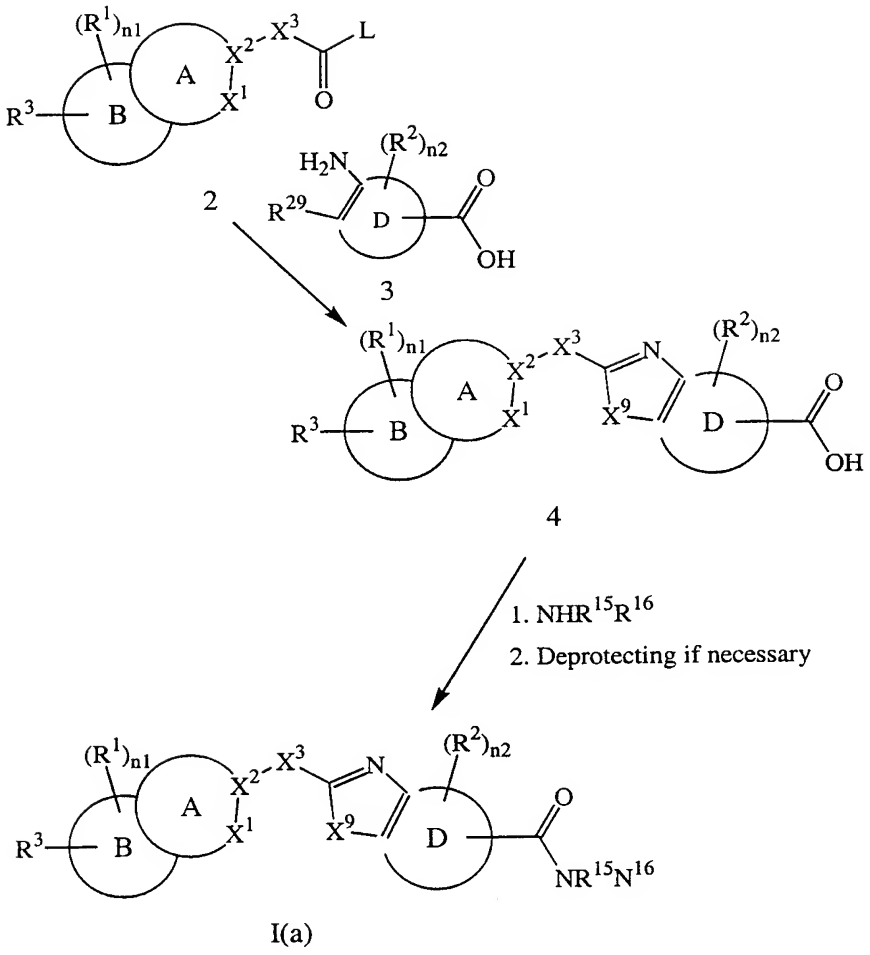
The amount of a compound of the invention in the composition may vary widely depending upon the type of formulation, size of a unit dosage, kind of excipients and other factors known to those of skill in the art of pharmaceutical sciences. In general, a composition of a compound of the invention for treating an infection will comprise from 0.01%w to 10%w, preferably 0.3%w to 1%w, of active ingredient with the remainder being the excipient or excipients. Preferably the pharmaceutical composition is administered in a single unit dosage form for continuous treatment or in a single unit dosage form ad libitum when relief of symptoms is specifically required. Representative pharmaceutical formulations containing a compound of the invention are described in Example 26.

Chemistry:

Generally, the compounds of the present invention are synthesized using standard techniques and reagents known to and used by those of skill in the art. It will be noted that the linkages between the various functional groups generally comprise carbon linked to the nitrogen of an amide or carbamate, the oxygen of a carbamate or the carbon of a carbonyl. Those of skill in the art will recognize that methods and reagents for forming these bonds are well known and readily available. *See*, *e.g.*, March, ADVANCED ORGANIC CHEMISTRY, 4th Ed. (Wiley 1992), Larock, COMPREHENSIVE ORGANIC TRANSFORMATIONS (VCH 1989); and Furniss, *et al.*, VOGEL'S TEXTBOOK OF PRACTICAL ORGANIC CHEMISTRY 5th ed. (Longman 1989), each of which is incorporated herein by reference.

In particular, compounds of Formula I in which X^4 and X^5 are adjacent members of an oxazol-2-yl, 1*H*-imidazol-2-yl or thiazol-2-yl ring and R^4 is $-C(O)N(R^{13})R^{12}$, can be prepared by the methods depicted in the following reaction scheme:

Scheme 1



I(a)

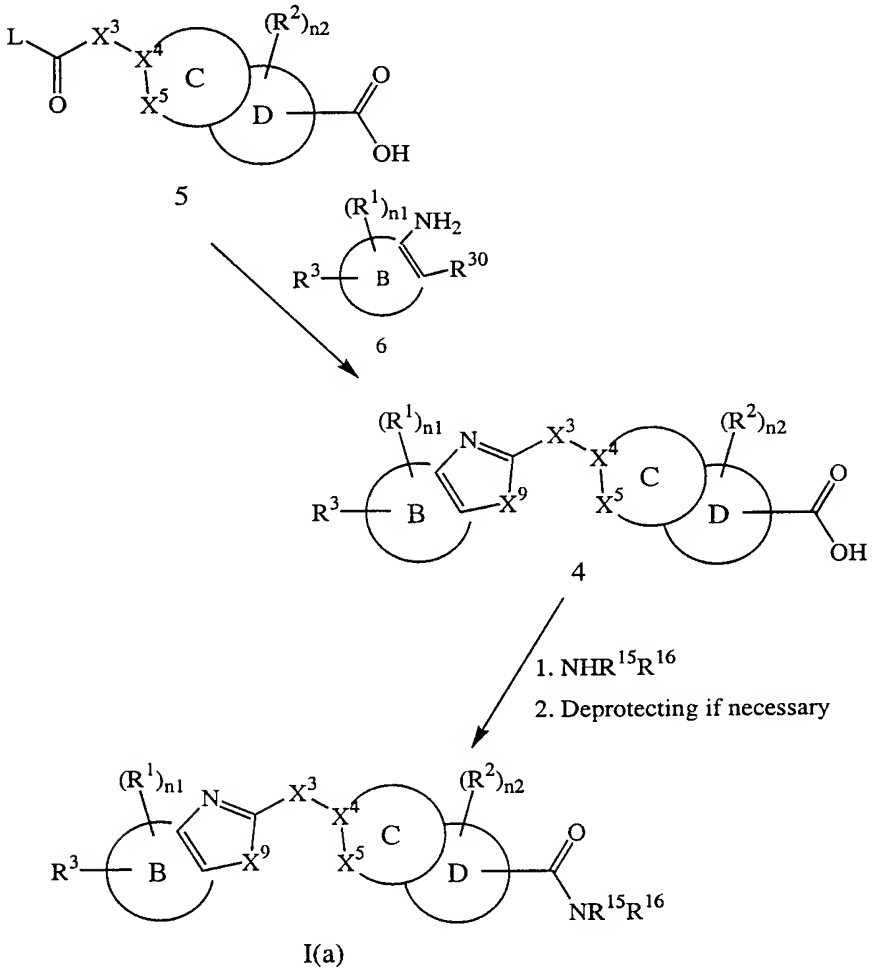
in which L is a leaving group, D together with the vinylene moiety to which it is fused comprise a monocyclic or fused bicyclic divalent radical containing from 5 to 15 annular atoms, wherein each ring contains 5 to 7 annular atoms and each annular atom optionally is a heteroatom, R^{29} is –OH, –NHR^6 or –SH, X^9 is –O–, –NR^6– or –S– and n2, n3, n4, A, B, X^1, X^2, X^3, X^5, R^1, R^2, R^3, R^4 and R^6 are as defined in the Summary of the Invention.

Compounds of Formula I in which X^4 and X^5 are adjacent members of an oxazol-2-yl, 1*H*-imidazol-2-yl or thiazol-2-yl ring (Formula I(a)) can be prepared by reacting a compound of Formula 1, or a protected derivative thereof, with a compound of Formula 2, or a protected derivative thereof, and then deprotecting if necessary. The reaction between the compounds

of Formulae 1 and 2 may be carried out neat, but preferably is carried out in the presence of 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone (DMPU) or polyphosphoric acid, at 160 to 200°C, preferably 180-190°C, and requires 1 to 5 hours to complete. Deprotection can be effected by any means which removes the protective group and gives the desired product in reasonable yield.

In a similar fashion, compounds of Formula I in which X^1 and X^2 adjacent members of an oxazol-2-yl, 1*H*-imidazol-2-yl or thiazol-2-yl ring can be prepared by the methods depicted in the following reaction scheme:

Scheme 2



in which L is a leaving group, R^{30} is –OH, –NHR^5 or –SH, X^9 is –O–, –NR^5– or –S– and n2, n3, n4, B, C, X^1, X^3, X^4, X^5, R^1, R^2, R^3, R^4 and R^6 are as defined in the Summary of the Invention.

Isolation and purification of the compounds and intermediates described herein can be effected, if desired, by any suitable separation or purification procedure such as, for example, filtration, extraction, crystallization, column chromatography, thin-layer chromatography or thick-layer chromatography, high-pressure liquid chromatography (HPLC), or a combination of these procedures. Specific illustrations of suitable separation and isolation procedures can be had by reference to the examples hereinbelow. However, other equivalent separation or isolation procedures can, of course, be used. Nuclear magnetic resonance (NMR) spectra were recorded on a General Electric "QE Plus" spectrometer (300 MHZ). Infrared (IR) spectra were recorded on a Perkin-Elmer 1600 Fourier Transform IR (FTIR). Analytical HPLC was performed on a Ultrafast Microprotein Analyzer, Michrom BioResources, Inc. equipped with a PLRP column, 1mm x 150mm. Preparative HPLC was performed on a Gilson LC using a VYDAC 1x25 cm C_{18} reverse phase (RP) column or a Waters Prep LC2000 system using a Vydac 5x25 cm C_{18} RP column. Mass spectra (MS) were obtained on a Finnigan SSQ 710 with an ESI source by direct infusion or by HPLC MS (Ultrafast Microprotein Analyzer, C_{18} column 2mm X 150 mm). Unless otherwise noted, all reagents and equipment were either prepared according to published procedures or were purchased from commercial sources, such as Aldrich Chemical Co. (Milwaukee, WI), Sigma Chemical Co. (St. Louis, MO) and ICN Chemical Co. (Irvine, CA). The techniques used to perform the syntheses described below will be recognized by those of skill in the art as routine (*see, e.g.*, March, Larock, or Furniss, *supra*).

Additional Processes for Preparing Compounds of the Invention:

Compounds of the invention in which R^3 is carbamoyl can be prepared by treating a compound of the invention in which R^3 is cyano with acid (e.g., hydrobromic acid) in a suitable solvent (e.g., acetic acid) for 5 to 8 hours at room temperature, then adding water to the reaction mixture and allowing 2 to 3 days for formation of the corresponding amide.

The compounds of the invention may be prepared as pharmaceutically acceptable acid

addition salts by reacting the free base forms of a compound of The invention with a pharmaceutically acceptable inorganic or organic acid. Alternatively, the pharmaceutically acceptable base addition salts of the compounds of the invention may be prepared by reacting the free acid forms of compounds of the invention with pharmaceutically acceptable inorganic or organic bases. Inorganic and organic acids and bases suitable for the preparation of the pharmaceutically acceptable salts of compounds of the invention are set forth in the definitions section of this application. Alternatively, the salt forms of the compounds of The invention may be prepared using salts of the starting materials or intermediates.

The free acid or free base forms of the compounds of The invention can be prepared from the corresponding base addition salt or acid addition salt form. For example, compounds of the invention in an acid addition salt form may be converted to the corresponding free base by treating with a suitable base (e.g., ammonium hydroxide solution, sodium hydroxide, etc.). Compounds of The invention in a base addition salt form may be converted to the corresponding free acid by treating with a suitable acid (e.g., hydrochloric acid, etc).

The *N*-oxides of compounds of the invention can be prepared by methods known to those of ordinary skill in the art. For example, *N*-oxides can be prepared by treating an unoxidized form of the compound of The invention with an oxidizing agent (e.g., trifluoroperacetic acid, permaleic acid, perbenzoic acid, peracetic acid, *meta*-chloroperoxybenzoic acid, etc.) in a suitable inert organic solvent (e.g., a halogenated hydrocarbon such as methylene chloride) at approximately 0°C. Alternatively, the *N*-oxides of the compounds of the invention can be prepared from the *N*-oxide of an appropriate starting material.

Compounds of the invention in unoxidized form can be prepared from *N*-oxides of compounds of The invention by treating with a reducing agent (e.g., sulfur, sulfur dioxide, triphenyl phosphine, lithium borohydride, sodium borohydride, phosphorus trichloride, tribromide, etc.) in an suitable inert organic solvent (e.g., acetonitrile, ethanol, aqueous dioxane, etc.) at 0 to 80°C.

Prodrug derivatives of the compounds of the invention can be prepared by methods

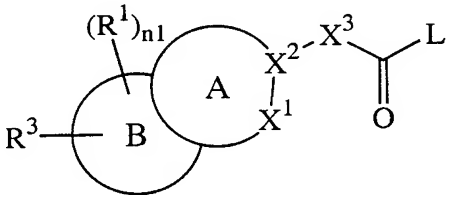
known to those of ordinary skill in the art. For further details on prodrugs and their preparation see Saulnier *et al.*(1994), *Bioorganic and Medicinal Chemistry Letters*. 4:1985)..

Protected derivatives of the compounds of the invention can be made by means known to those of ordinary skill in the art. A detailed description of the techniques applicable to the creation of protective groups and their removal can be found in T.W. Greene, *Protective Groups in Organic Synthesis*, John Wiley & Sons, Inc. 1981.

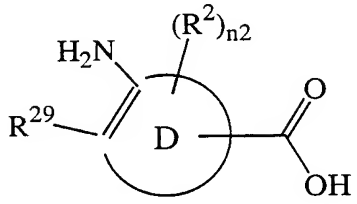
Compounds of the invention can be prepared as their individual stereoisomers by reacting a racemic mixture of the compound with an optically active resolving agent to form a pair of diastereoisomeric compounds, separating the diastereomers and recovering the optically pure enantiomer. While resolution of enantiomers can be carried out using covalent diasteromeric derivatives of compounds of the invention, dissociable complexes are preferred (e.g., crystalline diastereoisomeric salts). Diastereomers have distinct physical properties (e.g., melting points, boiling points, solubilities, reactivity, etc.) and can be readily separated by taking advantage of these dissimilarities. The diastereomers can be separated by chromatography or, preferably, by separation/resolution techniques based upon differences in solubility. The optically pure enantiomer is then recovered, along with the resolving agent, by any practical means that would not result in racemization. A more detailed description of the techniques applicable to the resolution of stereoisomers of compounds from their racemic mixture can be found in Jean Jacques Andre Collet, Samuel H. Wilen, Enantiomers, Racemates and Resolutions, John Wiley & Sons, Inc. (1981).

In summary, an aspect of this Invention is a process for preparing a compound of Formula I, which process comprises:

(a) reacting a compound of Formula 2:



or a protected derivative thereof, with a compound of Formula 3:



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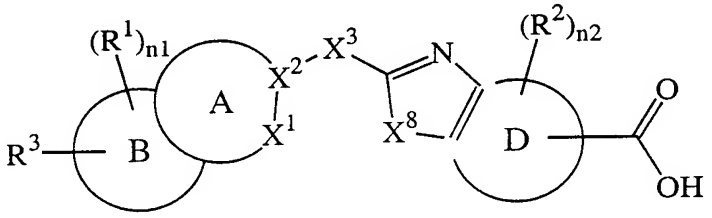
or a protected derivative thereof, in which L is a leaving group, D together with the vinylene moiety to which it is fused comprise a monocyclic or fused bicyclic divalent radical containing from 5 to 15 annular atoms, wherein each ring contains 5 to 7 annular atoms and each annular atom optionally is a heteroatom, R^{29} is –OH, –NHR^6 or –SH and n1, n2, n3, A, B, X^1, X^2, X^3, R^1, R^2, R^3, R^4 and R^6 are as defined in the Summary of the Invention, to give a compound of Formula 4:



4

and then reacting the compound of Formula 4 with an amine of the formula $NHR^{12}R^{13}$ and deprotecting if necessary to give a compound of Formula I in which X^4 and X^5 are adjacent members of an oxazol-2-yl, 1*H*-imidazol-2-yl or thiazol-2-yl ring and R^4 is –$C(O)N(R^{13})R^{12}$;

(c) optionally further converting a compound of Formula I into a pharmaceutically acceptable salt;

(d) optionally further converting a salt form of a compound of Formula I to non-salt form;

(e) optionally further converting an unoxidized form of a compound of Formula I into a

pharmaceutically acceptable *N*-oxide;

(f) optionally further an *N*-oxide form of a compound of Formula I its unoxidized form;

(g) optionally further converting a non-derivatized compound of Formula I into a pharmaceutically prodrug derivative; and

(h) optionally further converting a prodrug derivative of a compound of Formula I to its non-derivatized form.

Examples:

The following examples are provided merely for the purposes of illustration and are not to be construed in any way as limiting the scope of the present invention. Those skilled in the art will recognize that certain variations and modifications can be practiced within the scope of the invention.

EXAMPLE 1

3,4-Diamino-benzoic acid amide

3,4-Dinitro-benzoic acid amide (3.5 g, 16.6 mmol) was taken into methanol (100 mL and added to 10% palladium on carbon (1.0 g) under a nitrogen atmosphere. The mixture was then hydrogenated at 60 psi using a Parr apparatus over 8 hours. Filtration and concentration of the organic filtrate afforded 3,4-diamino-benzoic acid amide (2.5 g, 100%) as a tan solid; ^{1}H-NMR (300 MHZ, d_6-DMSO): 7.40 (br s, 1H), 7.05 (d, 1H), 6.95 (dd, 1H), 6.70 (br s, 1H), 6.45 (d, 1H), 4.90 (br s, 2H), 4.50 (br s, 2H).

EXAMPLE 2

3-Ethoxy-3-iminopropionic acid ethyl ester hydrochloride

A 2 L three-neck flask, equipped with drying tube, was charged with ethyl cyanoacetate (200 mL, 1.67 mol), toluene (1 L) and anhydrous ethanol (175 mL). The reaction mixture was cooled to 0° C and sparged with hydrogen chloride gas for 1 hour. The

reaction mixture was then sealed and allowed to warm to ambient temperature followed by stirring an additional 18 hours. The reaction mixture was diluted with an excess of ethyl ether (2 L) and the resulting precipitate was collected by filtration then washed with additional ethyl ether to afford 3-ethoxy-3-iminopropionic acid ethyl ester hydrochloride (270 g, 83%) as a white solid; ^{1}H-NMR (300 MHZ, d_6-DMSO): 7.69 (br s, 1H), 7.50 (br s, 1H), 4.50 (q, 2H), 4.11 (q, 2H), 3.98 (s, 2H), 1.30 (tr, 3H), 1.16 (tr, 3H).

EXAMPLE 3

(5-Carbamoyl-1*H*-benzoimidazol-2-yl)-acetic acid ethyl ester

3,4-diamino-benzoic acid amide (756 mg, 5.0 mmol), prepared as in Example 1, was taken into glacial acetic acid (5 mL) followed by addition of 3-ethoxy-3-iminopropionic acid ethyl ester hydrochloride (1.47 g, 7.5 mmol), prepared as in Example 2, and the mixture was warmed to 70°C for one hour. The mixture was concentrated *in vacuo* and the residue partitioned with saturated aqueous sodium hydrogen carbonate (10 mL) and ethyl acetate (10 mL). A solid formed and an excess of ethyl ether was added to the suspension. Filtration and washing the solid with additional ethyl ether afforded (5-carbamoyl-1*H*-benzoimidazol-2-yl)-acetic acid ethyl ester (803 mg, 65%) as a grey solid; ^{1}H-NMR (300 MHZ, d_6-DMSO): 8.10 (s, 1H), 7.95 (br s, 1H), 7.75 (d, 1H), 7.50 (d, 1H), 7.20 (br s, 1H), 4.10 (q, 2H), 4.00 (s, 2H), 1.20 (tr, 3H).

EXAMPLE 4

3-Methylamino-4-nitro-benzoic acid

To a sealable tube was added 4-nitro-3-methoxy-benzoic acid (5.0 g, 25.4 mmol) and aqueous methylamine (40%, 15 mL). The tube was securely capped and placed in an oil bath heated at 100 °C. A blast shield was used for safety. The reaction mixture was heated for 12 hours and allowed to cool to room temperature. The orange product was precipitated by pouring the crude mixture into a stirring slurry of 1 M aqueous hydrochloric acid and ice.

The product was filtered and rinsed with water. Recrystallization from hot ethanol afforded 3-methylamino-4-nitro-benzoic acid (3.6 g, 73%) as a bright red crystalline solid; ^{1}H-NMR (300 MHZ, d_6-DMSO): 13.5 (s, 1H), 8.3 (q, 1H), 8.2 (d, 1H), 7.4 (s, 1H), 7.1 (d, 1H), 3.0 (d, 3H).

EXAMPLE 5

3-Methylamino-4-amino-benzoic acid

3-Methylamino-4-nitro-benzoic acid (5.0 g, 25.5 mmol), prepared as in Example 4, was taken up into a 2:1 methanol and tetrahydrofuran solution (300 mL) and added to 10% palladium on carbon (1 g) under a nitrogen atmosphere. The mixture was hydrogenated at 60 psi using a Parr apparatus over 8 hours. Filtration followed by concentration of the organic solution afforded 3-methylamino-4-amino-benzoic acid (4.2 g, 100%) as a brown solid; ^{1}H-NMR (300 MHZ, d_6-DMSO): 7.10 (d, 1H), 6.90 (s, 1H), 6.50 (d, 1H), 5.25 (br s, 2H), 4.75 (br s, 1H), 2.65 (s, ,3H).

Example 6

2-(5-Carbamoyl-1*H*-benzoimidazol-2-ylmethyl)-3-methyl-3*H*-benzoimidazole-5-carboxylic acid

5-Carbamoyl-1*H*-benzoimidazol-2-yl-acetic acid ethyl ester (800 mg, 3.24 mmol), prepared as in Example 3, and 3-methylamino-4-amino-benzoic acid (540 mg, 3.24 mmol), prepared as in Example 5, were combined neat followed by addition of 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidone (DMPU, 1 g) and the mixture was heated to 190° C under a nitrogen atmosphere for 1.5 hours. The homogeneous liquid was cooled to ambient temperature followed by addition of an excess of dichloromethane. Agitation of the mixture with warming afforded a grey precipitate which was collected by filtration and washed with additional dichloromethane to afford 2-(5-carbamoyl-1*H*-benzoimidazol-2-ylmethyl)-3-methyl-3*H*-benzoimidazole-5-carboxylic acid (958 mg, 85%); ^{1}H-NMR (300 MHZ,

d_6-DMSO): 8.30 (s, 1H), 8.21 (s, 1H), 8.19 (br s, 1H), 7.95 (d, 1H), 7.90 (d, 1H), 7.75 (d, 1H), 7.70 (d, 1H), 7.45 (br s, 1H), 5.01 (s, 2H), 4.00 (s, 3H).

EXAMPLE 7

2-{[2-(5-Carbamoyl-1*H*-benzoimidazol-2-ylmethyl)-3-methyl-3*H*-benzoimidazole-5-carbonyl]-amino}-phosphono-propionic acid

(Compound 1)

2-Amino-3-phosphonopropionic acid (56 mg, 0.33 mmol) and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (1.0 mL, 5.4 mmol) were heated to 60° C under a nitrogen atmosphere for one hour to give a homogeneous solution of 2-amino-3-phosphonopropionic acid tris-trimethylsilyl ester. The solution was then concentrated *in vacuo* to a colorless oil and subsequently taken into DMF solution (0.5 mL). In a separate reaction vessel, 2-(5-carbamoyl-1*H*-benzoimidazol-2-ylmethyl)-3-methyl-3*H*-benzoimidazole-5-carboxylic acid (115.3 mg, 0.33 mmol), prepared as in Example 6, in DMF solution cooled to 0° C, was sequentially treated with bromo-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBroP, 153.8 mg, 0.33 mmol), diisopropylethylamine (0.12 mL, 0.67 mmol) and the previously generated DMF solution of 2-amino-3-phosphonopropionic acid tris-trimethylsilyl ester. The mixture was warmed to 40° C and stirred a total of 12 hours. Concentration *in vacuo* followed by treatment with trifluoroacetic acid (1 mL) afforded a homogeneous solution which was concentrated again. The residue was taken into 5% aqueous acetonitrile and the coupled product was purified by preparative reverse phase HPLC. Lyophillization of the pure fractions afforded 2-{[2-(5-carbamoyl-1*H*-benzoimidazol-2-ylmethyl)-3-methyl-3*H*-benzoimidazole-5-carbonyl]-amino}-phosphono-propionic acid (32 mg, 18%) as an off white amorphous solid; plasma desorption LRMS: Calculated for $C_{21}H_{22}N_6O_7P$ (MH^+): 501.4, Found: 501.0.

Proceeding as in Example 7 and substituting different starting materials the following compounds of the invention were prepared:

2-({2-[1-(5-fluoro-1*H*-benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid (Compound 2); plasma desorption LRMS: Calculated for $C_{21}H_{22}FN_5O_6P$ (MH^+): 490.4, Found: 490.0;

2-({2-[1-(5-hydroxy-1*H*-benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid (Compound 3); plasma desorption LRMS: Calculated for $C_{21}H_{23}N_5O_7P$ (MH^+): 488.4, Found: 488.5;

2-({2-[1-(5-chloro-1*H*-benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid (Compound 4); plasma desorption LRMS: Calculated for $C_{21}H_{22}ClN_5O_6P$ (MH^+): 506.9, Found: 506.2;

2-({2-[1-(1*H*-imidazol[4,5-*c*]pyridin-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid (Compound 5); plasma desorption LRMS: Calculated for $C_{20}H_{22}N_6O_6P$ (MH^+): 473.4, Found: 473.0;

2-({2-[1-(5-carbamoyl-1*H*-benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid (Compound 6); plasma desorption LRMS: Calculated for $C_{22}H_{24}N_6O_7P$ (MH^+): 515.4, Found: 515.2;

2-({2-[1-(5-methylcarbamoyl-1*H*-benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid (Compound 7); plasma desorption LRMS: Calculated for $C_{23}H_{26}N_6O_7P$ (MH^+): 529.5, Found: 529.2;

2-({2-[1-(1*H*-benzoimidazol-2-yl)-ethyl]-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid (Compound 8); plasma desorption LRMS: Calculated for $C_{20}H_{21}N_5O_6P$ (MH^+): 458.4, Found: 458.1;

2-({2-[1-(5-dimethylcarbamoyl-1*H*-benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid (Compound 9); plasma desorption LRMS: Calculated for $C_{24}H_{28}N_6O_7P$ (MH^+): 543.5, Found: 543.1;

3-phosphono-2-({2-[1-(4,6,7-trifluoro-1*H*-benzoimidazole-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-propionic acid (Compound 10); plasma desorption LRMS: Calculated for $C_{21}H_{20}F_3N_5O_6P$ (MH^+): 526.4, Found: 526.4;

3-phosphono-2-({2-[1-(5,6-difluoro-1*H*-benzoimidazole-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-propionic acid (Compound 11); plasma desorption

LRMS: Calculated for $C_{21}H_{21}F_2N_5O_6P$ (MH^+): 508.4, Found: 508.3;

3-phosphono-2-({2-[1-(5,6,7-trifluoro-1*H*-benzoimidazole-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-propionic acid (Compound 12); plasma desorption LRMS: $C_{21}H_{20}F_3N_5O_6P$ (MH^+): 526.4, Found: 526.6;

3-phosphono-2-({2-[1-(5,7-difluoro-1*H*-benzoimidazole-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-propionic acid (Compound 13); plasma desorption LRMS: $C_{21}H_{21}F_2N_5O_6P$ (MH^+): 508.4, Found: 508.2;

3-phosphono-2-({2-[1-(4,5,6,7-tetrafluoro-1*H*-benzoimidazole-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-propionic acid (Compound 14); plasma desorption LRMS: $C_{21}H_{19}F_4N_5O_6P$ (MH^+): 544.4, Found: 543.4; and

3-phosphono-2-{[2-(4,6,7-trifluoro-1*H*-benzoimidazole-2-ylmethyl)-3-methyl-3*H*-benzoimidazole-5-carbonyl]-amino}-propionic acid (Compound 15); plasma desorption LRMS: $C_{20}H_{18}F_3N_5O_6P$ (MH^+): 512.4, Found: 512.1.

EXAMPLE 8

2-Benzyloxycarbonylamino-3-(dimethoxy-phosphoryl)-propionic acid methyl ester

A cooled (0° C) mixture of 2-amino-3-phosphonopropionic acid (1.019 g, 6.0 mmol), water (5 mL), and ether (10 mL) was rapidly stirred and treated with benzyl chloroformate (1.3 mL, 9.0 mmol) and 6 N sodium hydroxide (4.5 mL) in four alternating portions. After 16 hours, the organic solvent was removed under reduced pressure, and the residue was added to stirring acetone/methanol (100 mL, 2:1 v/v). The white precipitate that formed was isolated by filtration, rinsed with acetone and dried, to afford the tri-sodium salt of 2-benzyloxycarbonylamino-3-phosphono-propionic acid (2.34 g) This material was dissolved in 0.5 M hydrochloric acid, the solvent was removed under reduced pressure, and the residue so obtained was used directly in the next step.

A suspension of the above material, 2-benzyloxycarbonylamino-3-phosphono-propionic acid, in anhydrous tetrahydrofuran (30 mL) was cooled (0° C) under nitrogen and treated with a diazomethane/ether solution just until a yellow color persisted. The mixture

was allowed to gradually warm to 20° C and stand for 12 hours. The mixture was cooled (0° C), quenched with acetic acid, and diluted with ether. The organic solution was washed with saturated $NaHCO_3$, NaCl, and dried (Na_2SO_4). The ether was removed under reduced pressure, to afford 2-benzyloxycarbonylamino-3-(dimethoxy-phosphoryl)-propionic acid methyl ester as a pale yellow oil (1.98 g, 95% yield based on two steps). $C_{14}H_{20}NO_7P$; MW calc. 345.1, found (ES) 346. ^{1}H-NMR (300 MHZ, DMSO-d_6) d: 7.85 (d, 1H), 7.35 (s, 5H), 5.10 (s, 2H), 4.35-4.25 (m, 1H), 3.75-3.50 (m, 9H), 2.30-2.20 (m, 2H).

EXAMPLE 9

2-Amino-3-(dimethoxy-phosphoryl)-propionic acid methyl ester hydrochloride

A mixture of 2-benzyloxycarbonylamino-3-(dimethoxy-phosphoryl)-propionic acid methyl ester (0.20 g, 0.58 mmol), prepared as in Example 8, and 10% palladium on carbon was suspended in methanol (10 mL) and acidified with 4 M hydrochloric acid in dioxane (0.2 mL). The mixture was hydrogenated at 1 atm for 2 hours. The catalyst was removed by filtration, and the solvent was removed under reduced pressure. The product, 2-amino-3-(dimethoxy-phosphoryl)-propionic acid methyl ester hydrochloride, was obtained as a clear colorless oil (0.13 g, 91% yield). $C_6H_{14}NO_5P\bullet HCl$; ^{1}H-NMR (300 MHZ, DMSO-d_6) d: 6.90 (br s, 3H), 4.00 (m, 1H), 3.80-3.55 (m, 9H), 2.30 (dd, 2H).

EXAMPLE 10

3,4-Diaminobenzonitrile

A mixture of 4-amino-3-nitrobenzonitrile (2.0 g, 12.3 mmol), anhydrous ethanol (20 mL), and 10% palladium on carbon (approx. 0.5 g), was hydrogenated at 1 atm for 2 hours. The catalyst was removed by filtration, and the solvent was removed under reduced pressure. The product, 3,4-diaminobenzonitrile, was obtained as a tan solid (1.55 g, 95% yield). $C_7H_7N_5$; ^{1}H-NMR (300 MHZ, DMSO-d_6) d: 6.77 (d, 1H), 6.72 (s, 1H), 6.50 (d, 1H), 5.43 (br s, 2H), 4.85 (br s, 2H).

EXAMPLE 11

5-Cyano-1*H*-benzoimidazol-2-yl-acetic acid ethyl ester

A solution of 3,4-diaminobenzonitrile (1.0 g, 7.5 mmol) and acetic acid (8 mL) was treated under nitrogen with ethoxycarbonimidoyl-acetic acid ethyl ester hydrochloride (1.62 g, 8.3 mmol) , and the mixture was warmed to 85° C. After 3 hours, the mixture was treated with another portion of ethoxy-carbonimidoyl-acetic acid ethyl ester hydrochloride (0.2 g, 1.0 mmol), and heating was continued for 2 hours. The mixture was cooled and poured over cracked ice. The slurry was adjusted to approximately pH 9 with conc. ammonium hydroxide, and a tan solid formed. After allowing the mixture to stir for 1 hours, the product was isolated by filtration, rinsed with water, and dried. 5-Cyano-1*H*-benzoimidazol-2-yl-acetic acid ethyl ester was obtained as a tan powder (1.52 g, 89% yield). $C_{12}H_{11}N_3O_2$; ^{1}H-NMR (300 MHZ, DMSO-d_6) d: 12.90 (br s, 1H), 8.15 (s, 1H), 7.75 (d, 1H), 7.60 (d, 1H), 4.20 (q, 2H), 4.10 (s, 2H), 1.25 (t, 3H).

EXAMPLE 12

2-(5-Cyano-1*H*-benzoimidazol-2-ylmethyl)-3-methyl-3*H*-benzoimidazole-5-carboxylic acid

A solution of 5-cyano-1*H*-benzoimidazol-2-yl-acetic acid ethyl ester (0.60 g, 2.6 mmol), prepared as in Example 11, and 4-amino-3-methylamino-benzoic acid (0.44 g, 2.6 mmol), prepared as in Example 5, in 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone (DMPU, 2 mL) was degassed briefly under reduced pressure, placed under nitrogen, and heated to 185° C for 3 hours. The resulting brown solution was diluted with an equivalent volume of ethyl acetate and added to stirring anhydrous ether. The tan solid that formed was isolated by filtration, rinsed with ether, and dried.
2-(5-Cyano-1*H*-benzoimidazol-2-ylmethyl)-3-methyl-3*H*-benzoimidazole-5-carboxylic acid was obtained as a tan powder (0.95 g crude) and used directly in the next step. $C_{18}H_{13}N_5O_2$; ^{1}H-NMR (300 MHZ, DMSO-d_6) d: 12.90 (br s, 1H), 8.15 (s, 1H), 8.03 (s, 1H), 7.80 (d, 1H), 7.68 (d, 1H), 7.60 (d, 1H), 7.52 (d, 1H), 4.68 (s, 2H), 3.90 (s, 3H).

EXAMPLE 13

2-{[2-(5-Cyano-1*H*-benzoimidazol-2-ylmethyl)-3-methyl-3*H*-benzoimidazole-5-carbonyl]-amino}-3-(dimethoxy-phosphoryl)-propionic acid methyl ester

(Compound 16)

A solution of 2-(5-cyano-1*H*-benzoimidazol-2-ylmethyl)-3-methyl-3*H*-benzoimidazole-5-carboxylic acid (0.17 g, 0.51 mmol), prepared as in Example 12, 2-amino-3-(dimethoxy-phosphoryl)-propionic acid methyl ester hydrochloride (0.13 g, 0.52 mmol), prepared as in Example 9, and HOBt (0.070 g, 0.52 mmol) in DMF (1.75 mL) was chilled (-40° C) under nitrogen. The mixture was treated with EDC (0.10 g, 0.52 mmol), and *N*,*N*-diisopropylethylamine (0.18 mL, 1.0 mmol), and allowed to gradually warm to 20° C. After 16 hours, the solvent was removed under reduced pressure. The residue was suspended in chloroform, washed with saturated $NaHCO_3$, NaCl, and dried (Na_2SO_4). The solvent was removed under reduced pressure, and the crude material was purified by silica gel chromatography using an isocratic eluant consisting of 90/10/1 chloroform/methanol/acetic acid. The appropriate fractions were pooled, the solvents were removed under reduced pressure and the product, 2-{[2-(5-cyano-1*H*-benzoimidazol-2-ylmethyl)-3-methyl-3*H*-benzoimidazole-5-carbonyl]-amino}-3-(dimethoxy-phosphoryl)-propionic acid methyl ester was obtained as a white residue (0.10 g, 37%). $C_{24}H_{25}N_6O_6P$: MW calc. 524.2, found (ES) 525.1. ^{1}H-NMR (300 MHZ, DMSO-d_6) d: 13.05 (br s, 1H), 8.88 (d, 1H), 8.15-8.10 (m, 2H), 7.75-6.98 (m, 4H), 4.60 (s, 3H), 3.85 (s, 3H), 3.70-3.55 (m, 9H), 2.60-2.50 (m, 2H).

EXAMPLE 14

2-{[2-(5-Carbamoyl-1*H*-benzoimidazol-2-ylmethyl)-3-methyl-3*H*-benzoimidazole-5-carbonyl]-amino}-3-phosphonopropionic acid

(Compound 1)

A solution of 2-{[2-(5-cyano-1*H*-benzoimidazol-2-ylmethyl)-3-methyl-3*H*-benzoimidazole-5-carbonyl]-amino}-3-(dimethoxy-phosphoryl)-propionic acid methyl

ester (0.090 g, 0.17 mmol), prepared as in Example 13, in acetic acid (1 mL) was treated with 40 wt % hydrobromic acid in acetic acid (5 mL). After 6 hours, water (2 mL) was added, and the mixture allowed to stand for 2 days at 20° C. An orange solid was obtained upon dropwise addition of the solution to stirring acetone. The solid was isolated by filtration, rinsed with acetone, and purified by C18 reversed-phase HPLC (2→16% MeCN/H_2O containing 20 mM Hydrochloric acid, over 50 min.). The product, 2-{[2-(5-carbamoyl-1*H*-benzoimidazol-2-ylmethyl)-3-methyl-3*H*-benzoimidazole-5-carbonyl]-amino}-3-phosphono-propionic acid hydrochloride, was obtained as a pale yellow powder (0.074 g, 83% yield). $C_{21}H_{21}N_6O_7P$: MW calc. 500.1, found (ES) 501.1. ^{1}H-NMR (300 MHZ, DMSO-d_6) d: 8.85 (d, 1H, J = 8.0 Hz), 8.39 (s, 1H), 8.25 (s, 1H), 7.95 (d, 1H, J = 8.4 Hz), 7.92 (d, 1H, J = 8.4 Hz), 7.85 (d, 2H, J = 207 Hz), 7.78 (m, 2H), 5.19 (s, 2H), 4.25 (m, 1H), 4.03 (s, 3H), 2.35-2.05 (m, 2H).

EXAMPLE 15

2-Ethoxycarbonimidoyl-propionic acid ethyl ester hydrochloride

Ethyl 2-cyanopropionate (100 g 0.29 mol) was dissolved in ethanol (65mL) and the solution cooled to 0° C followed by saturation with dry hydrogen chloride gas. The mixture was allowed to warm to room temperature and stir over 24 hours at which point the reaction was again cooled to 0° C and saturated with hydrogen chloride gas. The mixture was allowed to warm to room temperature and stirred another 24 hours. The imidate salt was precipitated by addition of ethyl ether:hexane (1:1), filtered and dried *in vacuo* to give 2-ethoxycarbonimidoyl-propionic acid ethyl ester hydrochloride (119.6 g 73% yield) as a white solid; ^{1}H-NMR (300 MHZ, DMSO-d_6): 12.05 (br s, 2H), 4.50 (q, 2H), 4.15 (m, 3H), 1.30 (m, 6H), 1.20 (tr, 3H).

EXAMPLE 16

2-(3*H*-Imidazo[4,5-*c*]pyridin-2-yl)-propionic acid ethyl ester hydrochloride

3,4-Diaminopyridine (20.0 g 184 mmol) and 2-ethoxycarbonimidoyl-propionic acid ethyl ester hydrochloride (46 g, 220 mmol), prepared as in Example 15, were taken up into glacial acetic acid (100 mL). The mixture was then heated to reflux with stirring for 1.5 hours. The crude material was concentrated *in vacuo* to a thick oil then diluted with ethyl acetate (1L). An insoluble amorphous residue was obtained and the solution was decanted. The ethyl acetate solution was partitioned with saturated aqueous sodium bicarbonate followed by addition of an excess of solid sodium bicarbonate sufficient to neutralize residual acetic acid. Solid sodium chloride was added in sufficient quantity to saturate the aqueous phase which was extracted with ethyl acetate 3x and the organic layers combined. The insoluble residue was then taken into a minimum of water and neutralized by addition of an excess of solid sodium bicarbonate and the aqueous mixture was extracted with ethyl acetate 1x. The organic layer was combined with the previously obtained ethyl acetate solution and the combined ethyl acetate solutions dried over anhydrous magnesium sulfate. Filtration and concentration afforded an orange oil (32g) which slowly crystallized. The hydrochloride salt is obtained by precipitation with ethyl ether and filtration to give 2-(3*H*-imidazo[4,5-*c*]pyridin-2-yl)-propionic acid ethyl ester hydrochloride (12.5 g) as a white hygroscopic solid; ^{1}H-NMR (300 MHZ, d_6-DMSO): 9.40 (s, 1H), 8.59 (d, 1H), 8.15 (d, 1H), 4.40 (q, 1H), 4.05-4.20 (m, 2H), 1.60 (d, 3H), 1.15 (tr, 3H).

EXAMPLE 17

2-(4,5,6,7-Tetrahydro-3*H*-imidazo[4,5-*c*]pyridin-2-yl)-propionic acid ethyl ester hydrochloride

2-(3*H*-Imidazo[4,5-*c*]pyridin-2-yl)-propionic acid ethyl ester hydrochloride (34.7 g 158 mmol), prepared as in Example 16, was dissolved in trifluoroacetic acid (50 mL) followed by addition of platinum oxide (2.5 g) and the mixture was hydrogenated at 50 psi in

a Parr hydrogenation apparatus for 24 hours. The suspension was filtered and the solution concentrated *in vacuo*. The oily residue was dissolved in a minimum of ethanol followed by addition of dry hydrogen chloride in dioxane solution (4M, 120 mL, 475 mmol). An excess of ethyl ether was added to the acidic solution and the resulting precipitate was collected by filtration and dried *in vacuo* to afford 2-(4,5,6,7-tetrahydro-3*H*-imidazo[4,5-*c*]pyridin-2-yl)-propionic acid ethyl ester hydrochloride as a hygroscopic white solid (30.7 g 66% yield); ^{1}H-NMR (300 MHZ, DMSO-d_6): 10.00 (br s, 2H), 4.35 (q, 1H), 4.20 (br s, 2H), 4.10 (m, 2H), 3.35 (m, 2H), 2.90 (br s, 2H), 1.55 (d, 3H), 1.15 (tr, 3H).

EXAMPLE 18

2-(1-Ethoxycarbonyl-ethyl)-1,4,6,7-tetrahydro-3*H*-imidazo[4,5-*c*]pyridine-5-carboxylic acid benzyl ester

2-(4,5,6,7-Tetrahydro-3*H*-imidazo[4,5-*c*]pyridin-2-yl)-propionic acid ethyl ester hydrochloride (60.2 g, 0.2 mol), prepared as in Example 18, was added to acetonitrile (500 mL) followed by diisopropylethylamine (100 mL, 0.6 mol) and the resulting suspension was cooled to 0° C. Benzyl chloroformate (58 mL, 0.4 mol) was added slowly with stirring and the mixture was slowly warmed to ambient temperature and stirred an additional 16 hours. The mixture was concentrated *in vacuo* followed by addition of ethyl ether (500 mL). The organic solution was washed with 0.1 M aqueous hydrochloric acid, saturated aqueous sodium hydrogen carbonate and saturated aqueous sodium chloride then dried over anhydrous sodium sulfate, filtered and the organic solution concentrated to provide a colorless oil. The oil was dissolved in ethanol (320 mL) and the resulting solution cooled to 0° C. Sodium ethoxide in ethanol solution (2.6 M, 85 mL, 0.22 mol) was slowly added to the solution and the mixture was stirred for one hour at 0° C. Hydrogen chloride solution in dioxane (4 M, 50 mL) was added to the solution and the mixture concentrated *in vacuo*. The residue was partitioned with ethyl acetate and saturated aqueous sodium hydrogen carbonate and the organic layer was then washed with saturated aqueous sodium chloride, dried over anhydrous sodium sulfate and filtered. The organic solution was then concentrated in vacuo to provide

2-(1-ethoxycarbonyl-ethyl)-1,4,6,7-tetrahydro-3*H*-imidazo[4,5-*c*]pyridine-5-carboxylic acid benzyl ester (52 g, 72%) as a yellow amorphous material; ^{1}H-NMR (300 MHZ, DMSO-d_6): 11.75 (br s, 1H), 7.30 (s, 5H), 5.10 (s, 2H), 4.40 (br s, 2H), 4.05 (m, 2H), 3.75 (q, 1H), 3.65 (br s, 2H), 1.40 (d, 3H), 1.15 (tr, 3H).

EXAMPLE 19

3-Methyl-2-[1-(4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridin-2-yl)-ethyl]-3*H*-benzoimidazole-5-carboxylic acid hydrochloride

Proceeding as in Example 6 and substituting 2-(1-ethoxycarbonyl-ethyl)-1,4,6,7-tetrahydro-3*H*-imidazo[4,5-*c*]pyridine-5-carboxylic acid benzyl ester, prepared as in Example 18, for 5-carbamoyl-1*H*-benzimidazol-2-yl-acetic acid ethyl ester affords cyclocondensation product 2-[1-(5-carboxy-1-methyl-1*H*-benzimidazol-2-yl)-ethyl]-1,4,6,7-tetrahydro-3*H*-imidazo[4,5-c]pyridine-5-carboxylic acid benzyl ester as a grey solid. 2-[1-(5-Carboxy-1-methyl-1*H*-benzoimidazol-2-yl)-ethyl]-1,4,6,7-tetrahydro-3*H*-imidazo[4,5-c]pyridine-5-carboxylic acid benzyl ester (911 mg, 2.0 mmol) was dissolved in methanol (25 mL) and the solution added to 10% palladium on carbon under a nitrogen atmosphere. The mixture was hydrogenated at 60 psi using a Parr apparatus for 12 hours. The mixture was then acidified to pH 2 by dropwise addition of 4 M hydrogen chloride in dioxane solution and filtered. The organic solution was concentrated *in vacuo* to afford 3-methyl-2-[1-(4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridin-2-yl)-ethyl]-3*H*-benzoimidazole-5-carboxylic acid hydrochloride (725 mg, 100%) as an orange solid; plasma desorption LRMS: Calculated for $C_{17}H_{20}N_5O_2$ (MH^+): 326.4, Found: 326.2.

EXAMPLE 20

2-({2-[1-(5-Acetyl-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridin-2-yl)ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid

(Compound 18)

3-Methyl-2-[1-(4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridin-2-yl)-ethyl]-3*H*-benzoimidazole-5-carboxylic acid hydrochloride (61 mg, 0.17 mmol), prepared as in Example 19, was taken up into DMF (1.0 mL) followed by addition of diisopropylethylamine (0.5 mmol, 0.09 mL) and the solution was cooled to 0° C. Acetic anhydride (0.17 mmol, 0.016 mL) was added and the mixture was stirred an additional 5 minutes at 0° C then warmed to ambient temperature and concentrated *in vacuo*. The residue was taken into methanol (0.5 mL) followed by addition of an excess of ethyl ether. The organic solution was decanted from the insoluble residue and the precipitation process was repeated once. The residue was then dried *in vacuo*, taken into DMF (1.0 mL) and the solution cooled to 0° C. Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBroP, 79 mg, 0.17 mmol) was added to the solution and stirred an addition 15 minutes at 0° C. Diisopropylethylamine (0.18 mL, 1.0 mmol) was added followed by 2-amino-3-phosphonopropionic acid (29 mg, 0.17 mmol) and the suspension was warmed to 55° C for 12 hours. The mixture was concentrated *in vacuo* then taken into 5% aqueous acetonitrile and the solution was filtered. The coupled product was purified by preparative reverse phase HPLC. Lyophillization of the pure fractions afforded 2-({2-[1-(5-acetyl-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridin-2-yl)ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid as a white amorphous solid; plasma desorption LRMS: Calculated for $C_{22}H_{28}N_6O_7P$ (MH^+): 519.5, Found: 519.3.

Proceeding as in Example 20 and substituting different reagents the following compounds of the invention were prepared:

2-({2-[1-(5-benzyloxycarbonyl-4,5,6,7-tetrahydro-

1*H*-imidazo[4,5-*c*]pyridin-2-yl)ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid (Compound 19); plasma desorption LRMS: $C_{28}H_{32}N_6O_8P$ (MH^+): 611.6, Found: 610.8;

2-({2-[1-(5-methylcarbamoyl-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridin-2-yl)ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid (Compound 20); plasma desorption LRMS: $C_{22}H_{28}N_7O_7P$ (MH^+): 534.5, Found: 534.2;

2-({2-[1-(5-dimethylcarbamoyl-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridin-2-yl)ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid (Compound 21); plasma desorption LRMS: $C_{23}H_{30}N_7O_7P$ (MH^+): 548.5, Found: 548.2;

2-({2-[1-(5-benzylcarbamoyl-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridin-2-yl)ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid (Compound 22); plasma desorption LRMS: $C_{28}H_{32}N_7O_7P$ (MH^+): 609.6, Found: 610.3;

2-({2-[1-(5-*tert*-butylcarbamoyl-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridin-2-yl)ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid (Compound 23); plasma desorption LRMS: $C_{25}H_{34}N_7O_7P$ (MH^+): 575.6, Found: 576.4; and

2-({2-[1-(5-hexylcarbamoyl-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridin-2-yl)ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid (Compound 24); plasma desorption LRMS: $C_{27}H_{38}N_7O_7P$ (MH^+): 603.6, Found: 604.6.

EXAMPLE 21

2-(1*H*-Benzoimidazol-2-yl)-propionic acid ethyl ester

o-Phenylenediamine (11.0 g, 0.10 mol) and 2-ethoxycarbonimidoyl-propionic acid ethyl ester (25.5 g, 0.12 mol), prepared as in Example 14, were combined in acetic acid (30 mL) with cooling sufficient to maintain a temperature of 20° C. The mixture was

allowed to stir for 3 hours, then poured over cracked ice. The slurry was brought to pH 10-11 with K_2CO_3, and stirred for 3 hours to allow for crystallization. The solid material was isolated by filtration, rinsed with water and dried. 2-(1*H*-benzoimidazol-2-yl)-propionic acid ethyl ester was obtained as a tan solid (20.55 g, 93%): ^{1}H-NMR (300 MHz, DMSO-d_6) d: 12.45 (br s, 1H), 7.50 (m, 2H), 7.15 (m, 2H), 4.10 (m, 3H), 1.52 (d, 3H), 1.12 (t, 3H).

EXAMPLE 22

2-[1-(1*H*-Benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carboxylic acid

A solution of 2-(1*H*-benzoimidazol-2-yl)-propionic acid ethyl ester (4.00 g, 18 mmol), prepared as in Example 21, and 4-amino-3-methylaminobenzoic acid (3.05 g, 18 mmol), prepared as in Example 5, in DMPU (9 mL) was heated at 185° C for 2h. The mixture was cooled and diluted with an equivalent volume of ethyl acetate, and then added gradually to stirring ether. The green precipitate which formed was isolated by filtration, rinsed with ether, and subsequently reprecipitated from warm methanol/ether. The new precipitate was isolated and dried. 2-[1-(1*H*-benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carboxylic acid was obtained as a pale green powder (3.33 g, 57%): ^{1}H-NMR (300 MHz, DMSO-d_6) d: 12.8 (br s, 1H), 12.4 (br s, 1H), 8.2 (s, 1H), 7.8 (d, 1H), 7.6 (d, 1H), 7.5 (m, 2H), 7.1 (m, 2H), 4.9 (q, 1H), 3.8 (s, 3H), 1.8 (d, 3H).

EXAMPLE 23

2-({2-[1-(1*H*-Benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-(dimethoxy-phosphoryl)-propionic acid methyl ester

(Compound 17)

A solution of 2-[1-(1*H*-Benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carboxylic acid (0.19 g, 0.59 mmol), prepared as in Example 22, 2-amino-3-(dimethoxy-phosphoryl)-propionic acid methyl ester hydrochloride (0.17 g, 0.69 mmol), prepared as in Example 9, and HOBt (0.088 g, 0.65 mmol) in DMF (2.4 mL) was chilled (-50° C) under

nitrogen. The mixture was treated with EDC (0.13 g, 0.68 mmol), and *N,N*-diisopropylethylamine (0.40 mL, 2.3 mmol), and allowed to gradually warm to 20° C. After 16 hours, the solvent was removed under reduced pressure. The residue was suspended in ethyl acetate, washed with saturated $NaHCO_3$, NaCl, and dried (Na_2SO_4). The solvent was removed under reduced pressure. 2-({2-[1-(1*H*-Benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-(dimethoxy-phosphoryl)-propionic acid methyl ester was obtained as a golden amorphous residue and used directly in the next step without further purification: $C_{24}H_{28}N_5O_6P$: MW calc. 513.2, found (ES) 514.2. ^{1}H-NMR (300 MHz, DMSO-d_6) d: 8.9 (d, 1H), 7.9 (s, 1H), 7.7 (d, 1H), 7.6 (d, 1H), 7.4 (m, 2H), 7.2 (m, 2H), 4.9 (q, 1H), 4.7 (m, 1H), 3.8 (s, 3H), 3.7-3.5 (m, 9H), 2.5-2.3 (m, 2H), 1.8 (d, 3H).

EXAMPLE 24

2-({2-[1-(1*H*-Benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid

(Compound 25)

The entire quantity of 2-({2-[1-(1*H*-benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-(dimethoxy-phosphoryl)-propionic acid methyl ester prepared in Example 23 was dissolved in acetic acid (2 mL) and treated with 40 wt% of hydrobromic acid in acetic acid (2 mL). After 5 hours, water (1 mL) was added and the mixture was allowed to stand for 12 hours. Another 40 wt % hydrobromic acid of 40 wt % in acetic acid (2 mL) was added, and the mixture allowed to stand 24 hours. The solution was added to stirring ethyl acetate, and a brown solid was isolated by filtration and rinsed with ethyl acetate. The material was purified by C18 reversed-phase HPLC (2→27% MeCN/H_2O containing 0.1% TFA, over 50 min.). Appropriate fractions were pooled, and the solvent was removed under reduced pressure. The product was re-lyophilized from 0.1 M Hydrochloric acid. 2-({2-[1-(1*H*-Benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid hydrochloride was obtained as a pale yellow powder (0.14 g, 45%): $C_{21}H_{22}N_5O_6P$: MW calc. 471.1, found (ES) 472.1. ^{1}H-NMR (300

MHz, DMSO-d_6) d: 8.74 (d, 1H, J = 9 Hz), 8.23 (s, 1 H), 7.80-7.60 (m, 4 H), 7.55-7.50 (m, 2H), 5.42 (q, 1H, J = 9 Hz), 4.60 (m, 1H), 4.00 (s, 3H), 2.30-2.10 (m, 2H), 1.96 (d, 3H, J = 9 Hz).

Proceeding as in Example 24 and sustituting different starting materials the following compounds of the invention were prepared:

2-({2-[1-(1*H*-benzoimidazole-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid methyl ester (Compound 26); ES LRMS: $C_{22}H_{25}N_5O_6P$ (MH^+): 486.2, Found: 486.1;

3-({2-[1-(1*H*-benzoimidazole-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid (Compound 27); ES LRMS: $C_{21}H_{23}N_5O_6P$ (MH^+): 472.1, Found: 472.1;

[2-({2-[1-(1*H*-benzoimidazole-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-ethyl]-phosphonic acid (Compound 28); ES LRMS: $C_{20}H_{23}N_5O_4P$ (MH^+): 428.1, Found: 428.1; and

(*R*)-2-({2-[1-(1*H*-benzoimidazole-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-sulfo-propionic acid (Compound 29); ES LRMS: $C_{21}H_{22}N_5O_6S$ (MH^+): 472.1, Found: 472.2.

EXAMPLE 25

In vitro HCV-NS3 Protease Inhibition Assay

A mixture of HCV NS3 protease (1 to 3 nM), NS3 cofactor NS4a (10 μM), $ZnCl_2$ (5 μM), Tris (50 mM; pH 7.5), glycerol (50%), TWEEN-20® (polyoxyethylenesorbitan monolaurate; 0.05%) and test compound (varying concentrations) was incubated for 15 minutes at room temperature (21 to 24 °C) in 96-well microtiter plates. The quenced fluorescence substrate acetyl-Asp-Glu-Asp(Edans)-Glu-Glu-Abu-Ψ[COO]-Ala-Ser-Lys(Dabcyl)-NH_2 (AnaSpec, Inc., San Jose, CA, U.S.A.) was added to a final concentration

of 1.5 μM. The hydrolysis of the fluorescent substrate was followed spectrophotometrically at 485 nanometers after excitation at 355 nanometers (Taliani, M., Bianchi, E., Narjes, F., Fossatelli, M., Urbani, C.S., De Francesco, R., and Pessi, A., (1996) *Anal. Biochem.* 240, 60-67).

The velocity of the NS3 catalyzed hydrolysis was determined from the linear portion of the progress curves using a fMax Microplate Reader (Molecular Devices, Sunnyvale, CA, U.S.A.) interfaced with a Macintosh PowerPC computer. Apparent inhibition constants (K_i) were calculated from the progress curves using the software package Batch K_i (Biokin Ltd., Madison, WI, (Kuzmic, P. (1996) *Anal. Biochem.* 237, 260273) which provides a parametric method for determining inhibitor potency using a transformation of a tight binding inhibition model (Morrison, J.F. (1969) *Biochem. Biophys. Acta* 185, 269-286).

Proceeding as described in Example 25 or by methods known to those of ordinary skill, the following compounds of the invention were tested for HCV-NS3 protease inhibitory activity:

(Compound 1, K_i = 0.062 μM), (Compound 2, K_i = 0.582 μM), (Compound 3, K_i = 0.745 μM), (Compound 6, K_i = 0.621 μM), (Compound 14, K_i = 0.822 μM) and (Compound 25, K_i = 0.233 μM).

EXAMPLE 26

The following are representative pharmaceutical formulations containing a compound of the invention.

ORAL FORMULATION

Compound of the Invention	10-100 mg
Citric Acid Monohydrate	105 mg
Sodium Hydroxide	18 mg
Flavoring	
Water	q.s. to 100 mL

INTRAVENOUS FORMULATION

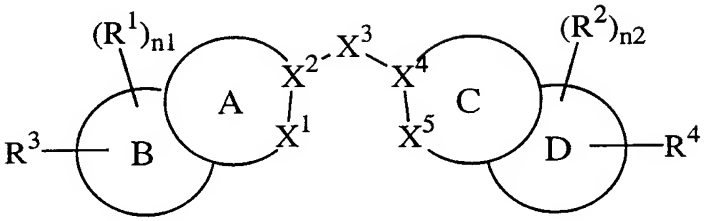
Compound of the Invention	0.1-10 mg
Dextrose Monohydrate	q.s. to make isotonic
Citric Acid Monohydrate	1.05 mg
Sodium Hydroxide	0.18 mg
Water for Injection	q.s. to 1.0 mL

TABLET FORMULATION

Compound of the Invention	1%
Microcrystalline Cellulose	73%
Stearic Acid	25%
Colloidal Silica	1%.

WE CLAIM:

1. A compound of Formula I:



I

in which:

n1 is 0, 1, 2, 3 or 4;

n2 is 0, 1, 2 or 3;

A together with B comprise a fused heterobicyclic radical containing 8 to 12 annular atoms, wherein each ring contains 5 to 7 annular members, each annular atom optionally is a heteroatom moiety, X^1 and X^2 are adjacent annular members of an aromatic ring and X^1 is a heteroatom moiety selected from –N=, –NR^5–, –O– and –S–, wherein R^5 is hydrogen or (C_{1-6})alkyl;

C together with D comprise a fused heterobicyclic radical containing 8 to 12 annular atoms, wherein each ring contains 5 to 7 annular members, each annular atom optionally is a heteroatom, X^4 and X^5 are adjacent annular members of an aromatic ring and X^5 is a heteroatom moiety selected from –N=, –NR^6–, –O– and –S–, wherein R^6 is hydrogen or (C_{1-8})alkyl optionally substituted with one to two substituents independently selected from halo, tri(C_{1-6})alkylammonio, –NR^7R^7, –C(O)NR^7R^7, –OR^7, –C(O)OR^7, –OC(O)R^7 or –$S(O)_2OR^7$, wherein R^7 at each occurrence independently is hydrogen or (C_{1-6})alkyl;

X^3 is –O–, –S–, –S(O)–, –$S(O)_2$–, –C(O)–, –NR^8– or –CR^8R^9–, wherein R^8 is hydrogen, halo, (C_{1-6})alkyl or together with R^9 forms (C_{2-6})alkylene or (C_{1-6})alkylidene and R^9 is hydrogen, halo, (C_{1-6})alkyl or as defined above, wherein any 1 to 3 carbon atoms with a free valence comprising R^8 and/or R^9 optionally independently are substituted with halo,

tri(C_{1-6})alkylammonio, $-NR^{10}R^{10}$, $-C(O)NR^{10}R^{10}$, $-OR^{10}$, $-C(O)OR^{10}$ or $-OC(O)R^{10}$, wherein R^{10} at each occurrence independently is hydrogen or (C_{1-6})alkyl;

R^1 at each occurrence independently is (C_{1-6})alkyl, (C_{1-6})alkyloxy, (C_{1-6})alkanoyloxy, (C_{1-6})alkylthio, halo, hydroxy or mercapto and bonded to any annular carbon atom with a free valence comprising B;

R^2 at each occurrence independently is (C_{1-6})alkyl, (C_{1-6})alkyloxy, (C_{1-6})alkanoyloxy, (C_{1-6})alkylthio, halo, hydroxy or mercapto and bonded to any annular carbon atom with a free valence comprising C;

R^3 is cyano, $-R^{11}$, $-CR^{12}R^{12}NR^{11}R^{13}$, $-C(NR^{13})R^{11}$, $-C(O)R^{11}$, $-C(NR^{13})NR^{11}R^{13}$, $-C(O)NR^{11}R^{13}$, $-C(O)OR^{11}$, $-S(O)R^{11}$, $-S(O)_2R^{11}$, $-S(O)_2NR^{11}R^{13}$ or $-S(O)_2OR^{11}$ and bonded to any annular atom with a free valence comprising B, wherein:

R^{11} is hydrogen, (C_{1-6})alkyl, cyclo(C_{3-6})alkyl(C_{0-3})alkyl, heterocyclo(C_{3-6})alkyl(C_{0-3})alkyl, (C_{6-10})aryl(C_{0-3})alkyl, hetero(C_{5-14})aryl(C_{0-3})alkyl, polycyclo(C_{9-10})aryl(C_{0-3})alkyl or heteropolycyclo(C_{8-10})aryl(C_{0-3})alkyl; wherein any alkyl moiety comprising R^{11} optionally independently is substituted with 1 to 3 substitutents selected from $-P(O)(OR^{14})OR^{14}$, $-S(O)_2OR^{14}$ and $-C(O)OR^{14}$ and any 1 to 3 annular carbon atoms with free valences of any aromatic ring comprising R^{11} optionally independently are substituted with halo, nitro, cyano, optionally halo-substituted (C_{1-6})alkyl, $-OR^{14}$, $-C(O)OR^{14}$, $-C(O)NR^{14}R^{14}$, $-X^6NR^{14}R^{14}$, $-X^6NR^{14}C(O)NR^{14}R^{14}$ or $-X^6NR^{14}C(NR^{14})NR^{14}R^{14}$, wherein X^6 is a bond or methylene and R^{14} at each occurrence independently is hydrogen or (C_{1-6})alkyl,

R^{12} at each occurrence independently is hydrogen, (C_{1-3})alkyl or together with another R^{12} and the carbon atom to which both are attached forms cyclopropyl and

R^{13} at each occurrence independently is hydrogen or (C_{1-6})alkyl; and

R^4 is $-R^{15}$, $-OR^{15}$, $-NR^{15}R^{16}$, $-SR^{15}$, $-S(O)R^{15}$, $-S(O)_2R^{15}$, $-S(O)_2OR^{15}$, $-S(O)_2NR^{15}R^{16}$, $-N(R^{16})S(O)_2R^{15}$, $-C(O)R^{15}$, $-C(O)OR^{15}$, $-C(O)NR^{15}R^{16}$, $-N(R^{16})C(O)R^{15}$, $-OC(O)NR^{15}R^{16}$, $-N(R^{16})C(O)OR^{15}$ or $-N(R^{16})C(O)NR^{15}R^{16}$, and bonded to any annular carbon atom with a free valence comprising C, wherein:

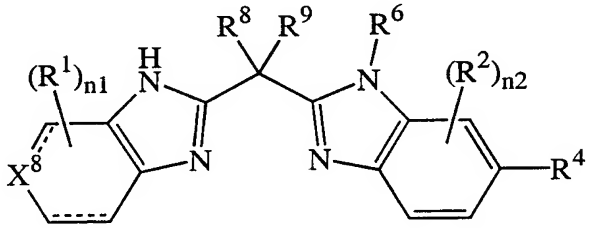
R^{15} is (C_{1-6})alkyl substituted with 1 to 2 radicals selected from

$-P(O)(OR^{17})OR^{17}$ and $-S(O)_2OR^{17}$ and optionally substituted with 1 to 2 $-C(O)OR^{17}$ groups, wherein R^{17} is hydrogen or (C_{1-6})alkyl, and

R^{16} is hydrogen or (C_{1-6})alkyl; and the *N*-oxide derivatives, prodrug derivatives, protected derivatives, individual isomers, mixtures of isomers and pharmaceutically acceptable salts thereof.

2. The compound of Claim 2 in which A together with B and C together with D comprise fused heterobicyclic radicals wherein A and C each contain 5 annular members and B and D each contain 6 annular members and X^1 and X^2 and X^4 and X^5 are adjacent members of an oxazol-2-yl, 1*H*-imidazol-2-yl or thiazol-2-yl ring; and the *N*-oxide derivatives, prodrug derivatives, protected derivatives, individual isomers, mixtures of isomers and pharmaceutically acceptable salts thereof.

3. A compound of Formula II:



II

in which:

the dashed lines independently represent optional bonds;

n1 is 0, 1, 2, 3 or 4;

n2 is 0, 1, 2 or 3;

X^8 is C, N, CR^3 or NR^3, wherein R^3 is cyano, (C_{1-6})alkyl, $-C(O)R^{11}$, $-C(O)NR^{11}R^{13}$ or $-C(O)OR^{11}$, wherein R^{11} independently is hydrogen, (C_{1-6})alkyl or (C_{1-4})aryl(C_{0-4})alkyl, R^{13} is hydrogen or (C_{1-6})alkyl and any alkyl moiety comprising R^{11} optionally independently is substituted with 1 to 3 substitutents selected from $-P(O)(OR^{14})OR^{14}$, $-S(O)_2OR^{14}$ and $-C(O)OR^{14}$, wherein R^{14} at each occurrence independently is hydrogen or (C_{1-6})alkyl;

provided that when X^8 is NR^3 the adjacent optional bond is not present and, unless indicated otherwise, any free valence of an annular atom is attached to a hydrogen atom;

R^1 and R^2 at each occurrence independently are (C_{1-6})alkyl, (C_{1-6})alkyloxy, halo or hydroxy and bonded to any annular carbon atom with a free valence;

R^4 is $-C(O)NR^{15}R^{16}$, wherein:

R^{15} is (C_{1-6})alkyl substituted with 1 to 2 radicals selected from $-P(O)(OR^{17})OR^{17}$ and $-S(O)_2OR^{17}$ and optionally substituted with 1 to 2 $-C(O)OR^{17}$ groups, wherein R^{17} is hydrogen or (C_{1-6})alkyl, and

R^{16} is hydrogen or (C_{1-6})alkyl;

R^6 is (C_{1-6})alkyl optionally substituted with one to two substituents independently selected from halo, tri(C_{1-6})alkylammonio, $-NR^7R^7$, $-C(O)NR^7R^7$, $-OR^7$, $-C(O)OR^7$, $-OC(O)R^7$ or $-S(O)_2OR^7$, wherein R^7 at each occurrence independently is hydrogen or (C_{1-6})alkyl; and

R^8 and R^9 independently are hydrogen, halo or (C_{1-6})alkyl, wherein any 1 to 3 carbon atoms with a free valence comprising R^8 and/or R^9 optionally independently are substituted with halo, tri(C_{1-6})alkylammonio, $-NR^{10}R^{10}$, $-C(O)NR^{10}R^{10}$, $-OR^{10}$, $-C(O)OR^{10}$ or $-OC(O)R^{10}$, wherein R^{10} at each occurrence independently is hydrogen or (C_{1-6})alkyl; and the *N*-oxide derivatives, prodrug derivatives, protected derivatives, individual isomers, mixtures of isomers and pharmaceutically acceptable salts thereof.

4. The compound of Claim 3 in which both of the optional bonds are present, n1and n2 each are 0, X^8 is N or CR^3, R^6 is (C_{1-4})alkyl, R^8 is hydrogen or methyl and R^9 is hydrogen; and the *N*-oxide derivatives, prodrug derivatives, protected derivatives, individual isomers, mixtures of isomers and pharmaceutically acceptable salts thereof.

5. The compound of Claim 4 in which R^3 is acetyl, benzyloxycarbonyl, cyano or $-C(O)NR^{11}R^{13}$, wherein R^{11} and R^{13} independently are hydrogen or methyl; and the *N*-oxide derivatives, prodrug derivatives, protected derivatives, individual isomers, mixtures of isomers and pharmaceutically acceptable salts thereof.

6. The compound of Claim 3 in which neither of the optional bonds are present, n1and n2 are 0, X^8 is NR^3, R^6 is (C_{1-4})alkyl, R^8 is hydrogen or methyl and R^9 is hydrogen; and the *N*-oxide derivatives, prodrug derivatives, protected derivatives, individual isomers, mixtures of isomers and pharmaceutically acceptable salts thereof.

7. The compound of Claim 6 in which R^3 is acetyl, benzyloxycarbonyl or $-C(O)NR^{11}R^{13}$, wherein R^{11} and R^{13} independently are hydrogen or methyl; and the *N*-oxide derivatives, prodrug derivatives, protected derivatives, individual isomers, mixtures of isomers and pharmaceutically acceptable salts thereof.

8. The compound of Claim 3 in which both of the optional bonds are present, n1 is 0, 1, 2, 3 or 4; n2 is 0; X^8 is C; R^1 at each occurrence is chloro, fluoro or hydroxy; R^6 is (C_{1-4})alkyl; R^8 is hydrogen or methyl; and R^9 is hydrogen; and the *N*-oxide derivatives, prodrug derivatives, protected derivatives, individual isomers, mixtures of isomers and pharmaceutically acceptable salts thereof.

9. The compound of Claim 5 in which X^8 is CR^3, wherein R^3 is carbamoyl, R^4 is 2-phosphono-1-carboxyethylcarbamoyl, R^6 is methyl and R^8 and R^9 are hydrogen, namely 2-{[2-(5-carbamoyl-1*H*-benzoimidazol-2-ylmethyl)-3-methyl-3*H*-benzoimidazole-5-carbonyl]-amino}-phosphono-propionic acid; and the *N*-oxide derivatives, prodrug derivatives, protected derivatives, individual isomers, mixtures of isomers and pharmaceutically acceptable salts thereof.

10. The compound of Claim 5 in which X^8 is CR^3, wherein R^3 is carbamoyl, R^4 is 2-phosphono-1-carboxyethylcarbamoyl, R^6 and R^8 are methyl and R^9 is hydrogen, namely 2-({2-[1-(5-carbamoyl-1*H*-benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid; and the *N*-oxide derivatives, prodrug derivatives, protected derivatives, individual isomers, mixtures of isomers and pharmaceutically acceptable salts thereof.

11. The compound of Claim 7 in which R^3 is methylcarbamoyl, R^4 is 2-phosphono-1-carboxyethylcarbamoyl, R^6 and R^8 are methyl and R^9 is hydrogen, namely 2-({2-[1-(5-methylcarbamoyl-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridin-2-yl)ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid; and the *N*-oxide derivatives, prodrug derivatives, protected derivatives, individual isomers, mixtures of isomers and pharmaceutically acceptable salts thereof.

12. The compound of Claim 8 in which n1 is 0, R^4 is 2-phosphono-1-carboxyethylcarbamoyl, R^6 and R^8 are methyl and R^9 is hydrogen, namely 2-({2-[1-(1*H*-benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid; and the *N*-oxide derivatives, prodrug derivatives, protected derivatives, individual isomers, mixtures of isomers and pharmaceutically acceptable salts thereof.

13. The compound of Claim 8 in which n1 is 1, R^1 is fluoro, R^4 is 2-phosphono-1-carboxyethylcarbamoyl, R^6 and R^8 are hydrogen and R^9 is hydrogen, namely 2-({2-[1-(5-fluoro-1*H*-benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid; and the *N*-oxide derivatives, prodrug derivatives, protected derivatives, individual isomers, mixtures of isomers and pharmaceutically acceptable salts thereof.

14. The compound of Claim 8 in which n1 is 1, R^1 is hydroxy, R^4 is 2-phosphono-1-carboxyethylcarbamoyl, R^6 and R^8 are methyl and R^9 is hydrogen, namely 2-({2-[1-(5-hydroxy-1*H*-benzoimidazol-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-3-phosphono-propionic acid; and the *N*-oxide derivatives, prodrug derivatives, protected derivatives, individual isomers, mixtures of isomers and pharmaceutically acceptable salts thereof.

15. The compound of Claim 8 in which n1 is 4, R^1 at each occurrence is fluoro, R^4

is 2-phosphono-1-carboxyethylcarbamoyl, R^6 and R^8 are methyl and R^9 is hydrogen, namely 3-phosphono-2-({2-[1-(4,5,6,7-tetrafluoro-1*H*-benzoimidazole-2-yl)-ethyl]-3-methyl-3*H*-benzoimidazole-5-carbonyl}-amino)-propionic acid; and the *N*-oxide derivatives, prodrug derivatives, protected derivatives, individual isomers, mixtures of isomers and pharmaceutically acceptable salts thereof.

16. A pharmaceutical composition which contains a compound of Claim 1 or a *N*-oxide derivative, prodrug derivative, individual isomer, mixture of isomers or pharmaceutically acceptable salt thereof in admixture with one or more suitable excipients.

17. A pharmaceutical composition which contains a compound of Claim 3 or a *N*-oxide derivative, prodrug derivative, individual isomer, mixture of isomers or pharmaceutically acceptable salt thereof in admixture with one or more suitable excipients.

18. A method of treating a patient infected with hepatitis C virus, which method comprises administering to the patient a therapeutically effective amount of compound of Claim 1 or a *N*-oxide derivative, prodrug derivative, individual isomer, mixture of isomers or pharmaceutically acceptable salt thereof.

19. A method of treating a patient infected with hepatitis C virus, which method comprises administering to the a patient therapeutically effective amount of compound of Claim 3 or a *N*-oxide derivative, prodrug derivative, individual isomer, mixture of isomers or pharmaceutically acceptable salt thereof.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/22850

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07D 235/04, 235/06, 235/20
US CL :514/394; 548/305.7, 335.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/394; 548/305.7, 335.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,693,515 A (CLARK et al) 02 December 1997, col. 5, lines 50-60 and col. 10, table 1.	1, 2 and 16
X	US 3,210,370 A (URSPRUNG, J.J.) 05 October 1965, col. 6, lines 5-14 and example 20.	1, 2 and 16
X,P	WO 98/452275 A1 (AXYS PHARMACEUTICALS CORPORATION.) 15 October 1998, see whole document.	1-5, 8-10, and 12-19

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 21 DECEMBER 1999	Date of mailing of the international search report 02 FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer CHANA AULAKH Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/22850

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
8, 12-15 and in part for 1-5, 9, 10 and 16-19

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/22850

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

I. Compounds of formula I where either ring A, B, C or D is a seven-membered ring containing atleast one N atom as the heteroatom, clasiified in class 540.

II. Compounds of formula I where either ring A, B, C or D is a 6-membered ring containing atleast one N atom as the heteroatom, classified in class 546.

III. Compounds of formula I where either ring A, B, C or D is a 5-7-membered ring containing only O or S as the heteroatoms, classified in class 549.

IV. Compounds of formula I where either ring A, B, C or D is a 5-membered ring containing only 3-4 nitrogens as heteroatoms, classified in class 548, subclass 250+.

V. Compounds of formula I where either ring A, B, C or D is a 5-membered ring containing atleast one N and one S or O as the heteroatoms, classified in class 548, subclass 100+.

VI. Compounds of formula I where either ring A, B, C or D is a 5-membered ring containing only 2 nitrogens as heteroatoms, classified in class 548, subclass 300.1+.

VII. Compounds of formula I where either ring A, B, C or D is a 5-membered ring containing only one N as the heteroatom, classified in class 548, subclass 400+.

The claims are deemed to correspond to the species listed above in the following manner:

Species VI : Claims 8 and 12-15.

Species II : Claims 6, 7 and 11.

The following claims are generic: 1-5, 9, 10 and 16-19.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons:
There is no common core which in the Markush Practice, is a significant structural element shared by all the alternatives; see PCT Administrative Instructions Annex B Part I (f) (i) (B) (1).

PCT WELTORGANISATION FÜR GEISTIGES EIGENTUM
Internationales Büro

INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

(51) Internationale Patentklassifikation [7] :
C07D 471/04, A61K 31/435 // (C07D 471/04, 235:00, 221:00)

A1

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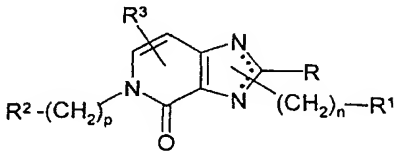
Veröffentlicht
Mit internationalem Recherchenbericht.
Vor Ablauf der für Änderungen der Ansprüche zugelassenen Frist; Veröffentlichung wird wiederholt falls Änderungen eintreffen.

(54) Title: IMIDAZO[4,5–C–]–PYRIDINE–4–ONE DERIVATIVES WITH FACTOR XA INHIBITING EFFECT

(54) Bezeichnung: IMIDAZO[4,5–C–]–PYRIDIN–4–ON–DERIVATE MIT FAKTOR XA HEMMENDER WIRKUNG



(I)

(57) Abstract

The invention relates to novel compounds of formula (I), wherein R, R^1, R^2, R^3, n and p have the meaning defined in claim 1. Said compounds are inhibitors of clotting factor Xa and can be used for the prophylaxis and/or therapy of thrombo–embolic disorders.

(57) Zusammenfassung

Neue Verbindungen der Formel (I), worin R, R^1, R^2, R^3, n und p die in Patentanspruch 1 angegebene Bedeutung haben, sind Inhibitoren des Koagulationsfaktors Xa und können zur Prophylaxe und/oder Therapie von thromboembolischen Erkrankungen eingesetzt werden.

LEDIGLICH ZUR INFORMATION

Codes zur Identifizierung von PCT-Vertragsstaaten auf den Kopfbögen der Schriften, die internationale Anmeldungen gemäss dem PCT veröffentlichen.

AL	Albanien	**ES**	Spanien	**LS**	Lesotho	**SI**	Slowenien
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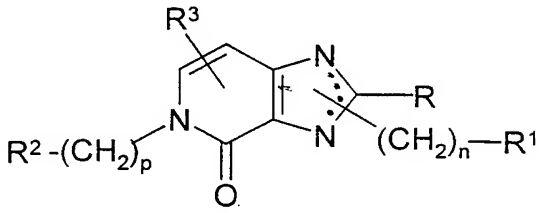
EE	Estland	**LR**	Liberia	**SG**	Singapur		

IMIDAZOL[4,5-C-]-PYRIDIN-4-ON-DERIVATE MIT FAKTOR XA HEMMENDER WIRKUNG

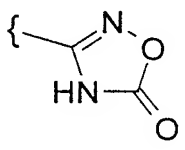
Die Erfindung betrifft Verbindungen der Formel I



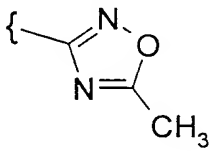
I

worin

R	H, unverzweigtes oder verzweigtes Alkyl mit 1-6 C-Atomen oder Cycloalkyl mit 3-6 C-Atomen,
R^1	Ar,
R^2	Ar',
R^3	H, R, R^4, Hal, CN, COOH, COOA oder $CONH_2$,
Ar, Ar'	jeweils unabhängig voneinander unsubstituiertes oder ein-, zwei- oder dreifach durch R, OH, Hal, CN, NO_2, CF_3, NH_2, NHR, NR_2, Pyrrolidin-1-yl, Piperidin-1-yl, Benzyloxy, SO_2NH_2, SO_2NHR, SO_2NR_2, -CONHR, $-CONR_2$, $-(CH_2)_n-NH_2$, $-(CH_2)_n-NHR$, $-(CH_2)_n-NR_2$, $-O-(CH_2)_n-NH_2$, $-O-(CH_2)_n-NHR$, $-O-(CH_2)_n-NR_2$, R^4 oder zusammen durch $-O-(CH_2)_m-O-$ substituiertes Phenyl, Naphthyl oder Biphenyl,
R^4	unsubstituiertes oder einfach durch -COR, -COOR, -OH oder durch eine konventionelle Aminoschutzgruppe substituiertes $-C(=NH)-NH_2$ oder $-NH-C(=NH)-NH_2$, $-C(=O)-N=C(NH_2)_2$,



oder



,

A	Alkyl mit 1-4 C-Atomen,
Hal	F, Cl, Br oder I,
m	1 oder 2,

n 0, 1, 2 oder 3,

p 0 oder 1 bedeutet,

sowie deren Salze.

Gegenstand der Erfindung sind auch die optisch aktiven Formen, die Racemate, die Diastereomeren sowie die Hydrate und Solvate, z.B. Alkoholate, dieser Verbindungen.

Der Erfindung lag die Aufgabe zugrunde, neue Verbindungen mit wertvollen Eigenschaften aufzufinden, insbesondere solche, die zur Herstellung von Arzneimitteln verwendet werden können.

Es wurde gefunden, daß die Verbindungen der Formel I und ihre Salze bei guter Verträglichkeit sehr wertvolle pharmakologische Eigenschaften besitzen. Insbesondere zeigen sie Faktor Xa inhibierende Eigenschaften und können daher zur Bekämpfung und Verhütung von thromboembolischen Erkrankungen wie Thrombose, myocardialem Infarkt, Arteriosklerose, Entzündungen, Apoplexie, Angina pectoris, Restenose nach Angioplastie und Claudicatio intermittens eingesetzt werden.

Die erfindungsgemäßen Verbindungen der Formel I können weiterhin Inhibitoren der Gerinnungsfaktoren Faktor VIIa, Faktor IXa und Thrombin der Blutgerinnungskaskade sein.

Aromatische Amidinderivate mit antithrombotischer Wirkung sind z.B. aus der EP 0 540 051 B1 bekannt. Cyclische Guanidine zur Behandlung thromboembolischer Erkrankungen sind z.B. in der WO 97/08165 beschrieben. Aromatische Heterocyclen mit Faktor Xa inhibitorischer Aktivität sind z.B. aus der WO 96/10022 bekannt. Substituierte N-[(Aminoiminomethyl)phenylalkyl]-azaheterocyclylamide als Faktor Xa Inhibitoren sind in WO 96/40679 beschrieben.

Der antithrombotische und antikoagulierende Effekt der erfindungsgemäßen Verbindungen wird auf die inhibierende Wirkung gegenüber der aktivierten Gerinnungsprotease, bekannt unter dem Namen Faktor Xa, oder

auf die Hemmung anderer aktivierter Serinproteasen wie Faktor VIIa, Faktor IXa oder Thrombin zurückgeführt.

Faktor Xa ist eine der Proteasen, die in den komplexen Vorgang der Blutgerinnung involviert ist. Faktor Xa katalysiert die Umwandlung von Prothrombin in Thrombin. Thrombin spaltet Fibrinogen in Fibrinmonomere, die nach Quervernetzung elementar zur Thrombusbildung beitragen. Eine Aktivierung von Thrombin kann zum Auftreten von thromboembolischen Erkrankungen führen. Eine Hemmung von Thrombin kann jedoch die in die Thrombusbildung involvierte Fibrinbildung inhibieren.
Die Messung der Inhibierung von Thrombin kann z.B. nach der Methode von G. F. Cousins et al. in *Circulation* **1996**, *94*, 1705-1712 erfolgen.

Eine Inhibierung des Faktors Xa kann somit verhindern, daß Thrombin gebildet wird.
Die erfindungsgemäßen Verbindungen der Formel I sowie ihre Salze greifen durch Inhibierung des Faktors Xa in den Blutgerinnungsprozeß ein und hemmen so die Entstehung von Thromben.

Die Inhibierung des Faktors Xa durch die erfindungsgemäßen Verbindungen und die Messung der antikoagulierenden und antithrombotischen Aktivität kann nach üblichen in vitro- oder in vivo-Methoden ermittelt werden. Ein geeignetes Verfahren wird z.B. von J. Hauptmann et al. in *Thrombosis and Haemostasis* **1990**, *63*, 220-223 beschrieben.

Die Messung der Inhibierung von Faktor Xa kann z.B. nach der Methode von T. Hara et al. in *Thromb. Haemostas.* **1994**, *71*, 314-319 erfolgen.

Der Gerinnungsfaktor VIIa initiiert nach Bindung an Tissue Faktor den extrinsischen Teil der Gerinnungskaskade und trägt zur Aktivierung des Faktors X zu Faktor Xa bei. Eine Inhibierung von Faktor VIIa verhindert somit die Entstehung des Faktors Xa und damit eine nachfolgende Thrombinbildung.
Die Inhibierung des Faktors VIIa durch die erfindungsgemäßen Verbindungen und die Messung der antikoagulierenden und antithrombotischen Aktivität kann nach üblichen in vitro- oder in vivo-Methoden ermittelt wer-

den. Ein übliches Verfahren zur Messung der Inhibierung von Faktor VIIa wird z.B. von H. F. Ronning et al. in *Thrombosis Research* **1996**, *84*, 73-81 beschrieben.

Der Gerinnungsfaktor IXa wird in der intrinsischen Gerinnungskaskade generiert und ist ebenfalls an der Aktivierung von Faktor X zu Faktor Xa beteiligt. Eine Inhibierung von Faktor IXa kann daher auf andere Weise verhindern, daß Faktor Xa gebildet wird.
Die Inhibierung von Faktor IXa durch die erfindungsgemäßen Verbindungen und die Messung der antikoagulierenden und antithrombotischen Aktivität kann nach üblichen in vitro- oder in vivo-Methoden ermittelt werden. Ein geeignetes Verfahren wird z.B. von J. Chang et al. in *Journal of Biological Chemistry* **1998**, *273*, 12089-12094 beschrieben.

Die Verbindungen der Formel I können als Arzneimittelwirkstoffe in der Human- und Veterinärmedizin eingesetzt werden, insbesondere zur Bekämpfung und Verhütung von thromboembolischen Erkrankungen wie Thrombose, myocardialem Infarkt, Arteriosklerose, Entzündungen, Apoplexie, Angina pectoris, Restenose nach Angioplastie und Claudicatio intermittens.

Gegenstand der Erfindung sind die Verbindungen der Formel I und ihre Salze sowie ein Verfahren zur Herstellung von Verbindungen der Formel I nach Anspruch 1 sowie ihrer Salze, dadurch gekennzeichnet, daß man

a) sie aus einem ihrer funktionellen Derivate durch Behandeln mit einem solvolysierenden oder hydrogenolysierenden Mittel in Freiheit setzt, indem man

   i) eine Amidinogruppe aus ihrem Oxadiazolderivat oder Oxazolidinonderivat durch Hydrogenolyse oder Solvolyse freisetzt,

   ii) eine konventionelle Aminoschutzgruppe durch Behandeln mit einem solvolysierenden oder hydrogenolysierenden Mittel durch Wasserstoff ersetzt oder eine durch eine konventionelle Schutzgruppe geschützte Aminogruppe in Freiheit setzt,

oder

b) in einer Verbindung der Formel I einen oder mehrere Rest(e) R, R^1, R^2 und/oder R^3 in einen oder mehrere Rest(e) R, R^1, R^2 und/oder R^3 umwandelt,

indem man beispielsweise

i) eine Estergruppe zu einer Carboxygruppe hydrolysiert,

ii) eine Nitrogruppe reduziert,

iii) eine Aminogruppe acyliert,

iv) eine Cyangruppe in eine Amidinogruppe

und/oder

c) eine Base oder Säure der Formel I in eines ihrer Salze umwandelt.

Für alle Reste, die mehrfach auftreten, gilt, daß deren Bedeutungen unabhängig voneinander sind.

Vor- und nachstehend haben die Reste bzw. Parameter R, R^1, R^2, R^3 und n die bei der Formel I angegebenen Bedeutungen, falls nicht ausdrücklich etwas anderes angegeben ist.

R bedeutet Alkyl, ist unverzweigt (linear) oder verzweigt, und hat 1 bis 6, vorzugsweise 1, 2, 3, 4, 5 oder 6 C-Atome. R bedeutet vorzugsweise Methyl, weiterhin Ethyl, Propyl, Isopropyl, Butyl, Isobutyl, sek.-Butyl oder tert.-Butyl, ferner auch Pentyl, 1-, 2- oder 3-Methylbutyl, 1,1- , 1,2- oder 2,2-Dimethylpropyl, 1-Ethylpropyl, Hexyl, 1- , 2- , 3- oder 4-Methylpentyl, 1,1- , 1,2- , 1,3- , 2,2- , 2,3- oder 3,3-Dimethylbutyl, 1- oder 2-Ethylbutyl, 1-Ethyl-1-methylpropyl, 1-Ethyl-2-methylpropyl, 1,1,2- oder 1,2,2-Trimethylpropyl. R ist auch Cycloalkyl und bedeutet vorzugsweise Cyclopropyl, Cyclobutyl, Cylopentyl, Cyclohexyl oder Cycloheptyl.

R bedeutet weiterhin H.

A bedeutet Alkyl mit 1, 2, 3 oder 4 C-Atomen und bedeutet vorzugsweise Methyl, weiterhin Ethyl, Propyl, Isopropyl, Butyl, Isobutyl, sek.-Butyl oder tert.-Butyl.

Hal bedeutet vorzugsweise F, Cl oder Br, aber auch I.

Ar und Ar' bedeuten jeweils unabhängig voneinander unsubstituiertes oder ein-, zwei- oder dreifach durch R, OH, OR, Hal, CN, NO_2, CF_3, NH_2, NHR, NR_2, Pyrrolidin-1-yl, Piperidin-1-yl, Benzyloxy, SO_2NH_2, SO_2NHA, SO_2NR_2, Phenylsulfonamido, $-(CH_2)_n-NH_2$, $-(CH_2)_n-NHR$, $-(CH_2)_n-NR_2$, $-O-(CH_2)_n-NH_2$, $-O-(CH_2)_n-NHR$, $-O-(CH_2)_n-NR_2$, $-O-(CH_2)_m-O-$ oder R^4 substituiertes Phenyl, Benzodioxol-5-yl, Naphthyl oder Biphenyl, wobei einfach durch Amidino substituiertes Naphthyl oder Biphenyl bevorzugt ist. Bevorzugte Substituenten für Biphenyl sind Amidino, Fluor, SO_2NH_2 oder SO_2NHR.

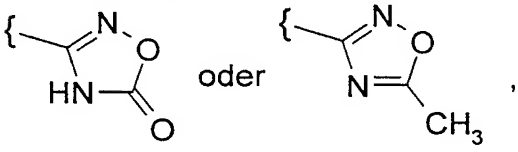
Ar und Ar' bedeuten jeweils unabhängig voneinander vorzugsweise unsubstituiertes Phenyl, Naphthyl oder Biphenyl, weiterhin vorzugsweise z.B. durch Methyl, Ethyl, Propyl, Isopropyl, Butyl, Cyclopentyl, Cyclohexyl, Fluor, Chlor, Brom, Iod, Hydroxy, Methoxy, Ethoxy, Propoxy, Butoxy, Pentyloxy, Hexyloxy, Cyan, Nitro, Trifluormethyl, Amino, Methylamino, Ethylamino, Dimethylamino, Diethylamino, Pyrrolidin-1-yl, Piperidin-1-yl, Benzyloxy, Sulfonamido, Methylsulfonamido, Ethylsulfonamido, Propylsulfonamido, Butylsulfonamido, Dimethylsulfonamido, Phenylsulfonamido, Aminomethyl, Aminoethyl, N-Methylaminomethyl, N-Ethylaminomethyl, N,N-Dimethylaminomethyl, Aminomethyloxy, Aminoethyloxy oder R^4 mono-, di- oder trisubstituiertes Phenyl, Naphthyl oder Biphenyl, ferner Benzodioxolyl.

Ar und Ar' bedeuten daher, jeweils unabhängig voneinander, ganz besonders bevorzugt z.B. o-, m- oder p-Tolyl, o-, m- oder p-Ethylphenyl, o-, m- oder p-Propylphenyl, o-, m- oder p-Isopropylphenyl, o-, m- oder p-tert.-Butylphenyl, o-, m- oder p-Hydroxyphenyl, o-, m- oder p-Nitrophenyl, o-, m- oder p-Aminophenyl, o-, m- oder p-(N-Methylamino)-phenyl, o-, m- oder p-(N-Methylaminocarbonyl)-phenyl, o-, m- oder p-Acetamidophenyl, o-, m- oder p-Methoxyphenyl, o-, m- oder p-Ethoxyphenyl, o-, m- oder p-(N,N-Dimethylamino)-phenyl, o-, m- oder p-(N,N-Dimethylaminocarbonyl)-phenyl,

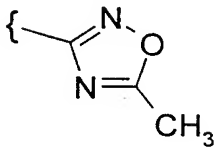
o-, m- oder p-(N-Ethylamino)-phenyl, o-, m- oder p-(N,N-Diethylamino)-phenyl, o-, m- oder p-Fluorphenyl, o-, m- oder p-Bromphenyl, o-, m- oder p- Chlorphenyl, o-, m- oder p-(Methylsulfonamido)-phenyl, o-, m- oder p-Amidinophenyl, 7-Amidino-2-naphthyl, 2'-Amidino-biphenyl-3-yl, 3-Fluor-2'-sulfamoyl-biphenyl-4-yl, 3-Fluor-2'-N-tert.-butyl-sulfamoyl-biphenyl-4-yl, 2'-Sulfamoyl-biphenyl-4-yl, 2'-N-tert.-Butyl-sulfamoyl-biphenyl-4-yl, o-, m- oder p-(Pyrrolidin-1-yl)-phenyl, o-, m- oder p-(Piperidin-1-yl)-phenyl, o-, m- oder p-{5-Methyl-[1,2,4]-oxadiazol-3-yl)}-phenyl, 7-{5-Methyl-[1,2,4]-oxadiazol-3-yl)}-naphth-2-yl, o-, m- oder p-{5-Oxo-[1,2,4]-oxadiazol-3-yl)}-phenyl, 7-{5-Oxo-[1,2,4]-oxadiazol-3-yl)}-naphth-2-yl, weiter bevorzugt 2,3-, 2,4-, 2,5-, 2,6-, 3,4- oder 3,5-Difluorphenyl, 2,3-, 2,4-, 2,5-, 2,6-, 3,4- oder 3,5-Dichlorphenyl, 2,3-, 2,4-, 2,5-, 2,6-, 3,4- oder 3,5-Dibromphenyl, 2,4- oder 2,5-Dinitrophenyl, 2,5- oder 3,4-Dimethoxyphenyl, 3-Nitro-4-chlor-phenyl, 3-Amino-4-chlor-, 2-Amino-3-chlor-, 2-Amino-4-chlor-, 2-Amino-5-chlor- oder 2-Amino-6-chlorphenyl, 2-Nitro-4-N,N-dimethylamino- oder 3-Nitro-4-N,N-dimethylaminophenyl, 2,3-Diaminophenyl, 2,3,4-, 2,3,5-, 2,3,6-, 2,4,6- oder 3,4,5-Trichlorphenyl, 2,4,6-Trimethoxyphenyl, 2-Hydroxy-3,5-dichlorphenyl, p-Iodphenyl, 3,6-Dichlor-4-aminophenyl, 4-Fluor-3-chlorphenyl, 2-Fluor-4-bromphenyl, 2,5-Difluor-4-bromphenyl, 3-Brom-6-methoxyphenyl, 3-Chlor-6-methoxyphenyl, 3-Chlor-4-acetamidophenyl, 3-Fluor-4-methoxyphenyl, 3-Amino-6-methylphenyl, 3-Chlor-4-acetamidophenyl oder 2,5-Dimethyl-4-chlorphenyl.

R^3 bedeutet vorzugsweise z.B. H, Hal, COOH, COOA oder $CONH_2$.

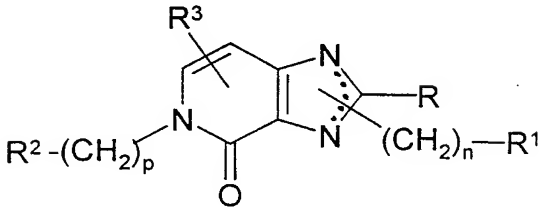
R^4 bedeutet vorzugsweise z.B. unsubstituiertes $-C(=NH)-NH_2$, $-NH-C(=NH)-NH_2$, $-C(=O)-N=C(NH_2)_2$, das auch einfach durch OH substituiert sein kann,

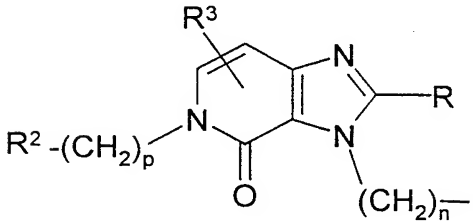


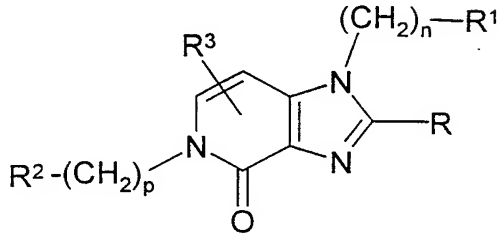
ganz besonders bevorzugt unsubstituiertes $-C(=NH)-NH_2$ oder



m bedeutet 1 oder 2.
n bedeutet vorzugsweise 0 oder 1, ferner auch 2 oder 3.

 bedeutet

 (IA)

oder  (IB).

Die Verbindungen der Formel I können ein oder mehrere chirale Zentren besitzen und daher in verschiedenen stereoisomeren Formen vorkommen. Die Formel I umschließt alle diese Formen.

Dementsprechend sind Gegenstand der Erfindung insbesondere diejenigen Verbindungen der Formel I, in denen mindestens einer der genannten Reste eine der vorstehend angegebenen bevorzugten Bedeutungen hat. Einige bevorzugte Gruppen von Verbindungen können durch die folgenden Teilformeln Ia bis Ii ausgedrückt werden, die der Formel I entsprechen und worin die nicht näher bezeichneten Reste die bei der Formel I angegebene Bedeutung haben, worin jedoch

in Ia Ar einfach durch R^4 substituiertes Phenyl, Naphthyl oder Biphenyl,

bedeutet;

in Ib Ar' einfach durch R^4 substituiertes Phenyl, Naphthyl oder Biphenyl,

bedeutet;

in Ic Ar, Ar' jeweils unabhängig voneinander einfach durch R^4 substituiertes Phenyl, Naphthyl oder Biphenyl,

bedeutet;

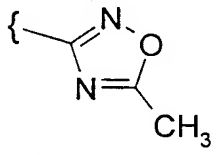
in Id Ar, Ar' jeweils unabhängig voneinander einfach durch $-CONR_2$, SO_2NH_2 oder R^4 substituiertes Phenyl, Naphthyl oder Biphenyl,

bedeutet;

in Ie R^3 H, R, Hal, COOH oder COOA,

bedeutet;

in If R^4 $-C(=NH)-NH_2$ oder



bedeutet;

in Ig n 1

bedeutet;

in Ih R H, unverzweigtes oder verzweigtes Alkyl mit 1-6 C-Atomen oder Cycloalkyl mit 3-6 C-Atomen,

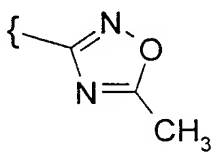
R^1 Ar,

R^2 Ar',

R^3 H, R, Hal, COOH oder COOA,

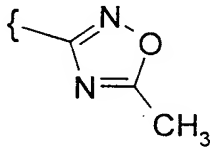
Ar, Ar' jeweils unabhängig voneinander einfach durch $-CONR_2$, SO_2NH_2 oder R^4 substituiertes Phenyl, Naphthyl oder Biphenyl,

R^4 $-C(=NH)-NH_2$ oder



,

	A	Alkyl mit 1-4 C-Atomen,
	Hal	F, Cl, Br oder I,
	m	1 oder 2,
	n	0, 1, 2 oder 3
	bedeutet,	
in Ii	R	H, unverzweigtes oder verzweigtes Alkyl mit 1-6 C-Atomen oder Cycloalkyl mit 3-6 C-Atomen,
	R^1	Ar,
	R^2	Ar',
	R^3	H, R, Hal, COOH oder COOA,
	Ar, Ar'	jeweils unabhängig voneinander einfach durch R^4 substituiertes Phenyl, Naphthyl oder Biphenyl,
	R^4	$-C(=NH)-NH_2$ oder



,

	A	Alkyl mit 1-4 C-Atomen,
	Hal	F, Cl, Br oder I,
	m	1 oder 2,
	n	0, 1, 2 oder 3,
	p	0 oder 1
	bedeutet.	

Die Verbindungen der Formel I und auch die Ausgangsstoffe zu ihrer Herstellung werden im übrigen nach an sich bekannten Methoden hergestellt, wie sie in der Literatur (z.B. in den Standardwerken wie Houben-Weyl, Methoden der organischen Chemie, Georg-Thieme-Verlag, Stuttgart) beschrieben sind, und zwar unter Reaktionsbedingungen, die für die genannten Umsetzungen bekannt und geeignet sind. Dabei kann man auch von an sich bekannten, hier nicht näher erwähnten Varianten Gebrauch machen.

Die Ausgangsstoffe können, falls erwünscht, auch in situ gebildet werden, so daß man sie aus dem Reaktionsgemisch nicht isoliert, sondern sofort weiter zu den Verbindungen der Formel I umsetzt.

Verbindungen der Formel I können vorzugsweise erhalten werden, indem man Verbindungen der Formel I aus einem ihrer funktionellen Derivate durch Behandeln mit einem solvolysierenden oder hydrogenolysierenden Mittel in Freiheit setzt.

Bevorzugte Ausgangsstoffe für die Solvolyse bzw. Hydrogenolyse sind solche, die sonst der Formel I entsprechen, aber anstelle einer oder mehrerer freier Amino- und/oder Hydroxygruppen entsprechende geschützte Amino- und/oder Hydroxygruppen enthalten, vorzugsweise solche, die anstelle eines H-Atoms, das mit einem N-Atom verbunden ist, eine Aminoschutzgruppe tragen, insbesondere solche, die anstelle einer HN-Gruppe eine R'-N-Gruppe tragen, worin R' eine Aminoschutzgruppe bedeutet, und/oder solche, die anstelle des H-Atoms einer Hydroxygruppe eine Hydroxyschutzgruppe tragen, z.B. solche, die der Formel I entsprechen, jedoch anstelle einer Gruppe -COOH eine Gruppe -COOR" tragen, worin R" eine Hydroxyschutzgruppe bedeutet.
Bevorzugte Ausgangsstoffe sind auch die Oxadiazolderivate, die in die entsprechenden Amidinoverbindungen überführt werden können.

Die Freisetzung der Amidinogruppe aus ihrem Oxadiazolderivat kann z.B. durch Behandeln mit Wasserstoff in Gegenwart eines Katalysators (z.B. Raney-Nickel) erfolgen. Als Lösungsmittel eignen sich die nachfolgend angegebenen, insbesondere Alkohole wie Methanol oder Ethanol, organische Säuren wie Essigsäure oder Propionsäure oder Mischungen daraus. Die Hydrogenolyse wird in der Regel bei Temperaturen zwischen etwa 0 und 100° und Drucken zwischen etwa 1 und 200 bar, bevorzugt bei 20-30° (Raumtemperatur) und 1-10 bar durchgeführt.

Die Einführung der Oxadiazolgruppe gelingt z.B. durch Umsetzung der Cyanverbindungen mit Hydroxylamin und Reaktion mit Phosgen, Dialkylcarbonat, Chlorameisensäureester, N,N'-Carbonyldiimidazol oder Acetanhydrid.

Es können auch mehrere - gleiche oder verschiedene - geschützte Amino- und/oder Hydroxygruppen im Molekül des Ausgangsstoffes vorhanden sein. Falls die vorhandenen Schutzgruppen voneinander verschieden sind, können sie in vielen Fällen selektiv abgespalten werden.

Der Ausdruck "Aminoschutzgruppe" ist allgemein bekannt und bezieht sich auf Gruppen, die geeignet sind, eine Aminogruppe vor chemischen Umsetzungen zu schützen (zu blockieren), die aber leicht entfernbar sind, nachdem die gewünschte chemische Reaktion an anderen Stellen des Moleküls durchgeführt worden ist. Typisch für solche Gruppen sind insbesondere unsubstituierte oder substituierte Acyl-, Aryl-, Aralkoxymethyl- oder Aralkylgruppen. Da die Aminoschutzgruppen nach der gewünschten Reaktion (oder Reaktionsfolge) entfernt werden, ist ihre Art und Größe im übrigen nicht kritisch; bevorzugt werden jedoch solche mit 1-20, insbesondere 1-8 C-Atomen. Der Ausdruck "Acylgruppe" ist im Zusammenhang mit dem vorliegenden Verfahren in weitestem Sinne aufzufassen. Er umschließt von aliphatischen, araliphatischen, aromatischen oder heterocyclischen Carbonsäuren oder Sulfonsäuren abgeleitete Acylgruppen sowie insbesondere Alkoxycarbonyl-, Aryloxycarbonyl- und vor allem Aralkoxycarbonylgruppen. Beispiele für derartige Acylgruppen sind Alkanoyl wie Acetyl, Propionyl, Butyryl; Aralkanoyl wie Phenylacetyl; Aroyl wie Benzoyl oder Toluyl; Aryloxyalkanoyl wie POA; Alkoxycarbonyl wie Methoxycarbonyl, Ethoxycarbonyl, 2,2,2-Trichlorethoxycarbonyl, BOC (tert.-Butyloxycarbonyl), 2-Iodethoxycarbonyl; Aralkyloxycarbonyl wie CBZ ("Carbobenzoxy"), 4-Methoxybenzyloxycarbonyl, FMOC; Arylsulfonyl wie Mtr. Bevorzugte Aminoschutzgruppen sind BOC und Mtr, ferner CBZ, Fmoc, Benzyl und Acetyl.

Der Ausdruck "Hydroxyschutzgruppe" ist ebenfalls allgemein bekannt und bezieht sich auf Gruppen, die geeignet sind, eine Hydroxygruppe vor chemischen Umsetzungen zu schützen, die aber leicht entfernbar sind, nachdem die gewünschte chemische Reaktion an anderen Stellen des Moleküls durchgeführt worden ist. Typisch für solche Gruppen sind die oben genannten unsubstituierten oder substituierten Aryl-, Aralkyl- oder Acylgruppen, ferner auch Alkylgruppen. Die Natur und Größe der Hydroxy-

schutzgruppen ist nicht kritisch, da sie nach der gewünschten chemischen Reaktion oder Reaktionsfolge wieder entfernt werden; bevorzugt sind Gruppen mit 1-20, insbesondere 1-10 C-Atomen. Beispiele für Hydroxyschutzgruppen sind u.a. Benzyl, 4-Methoxybenzyl, p-Nitrobenzoyl, p-Toluolsulfonyl, tert.-Butyl und Acetyl, wobei Benzyl und tert.-Butyl besonders bevorzugt sind.

Das In-Freiheit-Setzen der Verbindungen der Formel I aus ihren funktionellen Derivaten gelingt - je nach der benutzten Schutzgruppe - z. B. mit starken Säuren, zweckmäßig mit TFA oder Perchlorsäure, aber auch mit anderen starken anorganischen Säuren wie Salzsäure oder Schwefelsäure, starken organischen Carbonsäuren wie Trichloressigsäure oder Sulfonsäuren wie Benzol- oder p-Toluolsulfonsäure. Die Anwesenheit eines zusätzlichen inerten Lösungsmittels ist möglich, aber nicht immer erforderlich. Als inerte Lösungsmittel eignen sich vorzugsweise organische, beispielsweise Carbonsäuren wie Essigsäure, Ether wie Tetrahydrofuran oder Dioxan, Amide wie DMF, halogenierte Kohlenwasserstoffe wie Dichlormethan, ferner auch Alkohole wie Methanol, Ethanol oder Isopropanol, sowie Wasser. Ferner kommen Gemische der vorgenannten Lösungsmittel in Frage. TFA wird vorzugsweise im Überschuß ohne Zusatz eines weiteren Lösungsmittels verwendet, Perchlorsäure in Form eines Gemisches aus Essigsäure und 70 %iger Perchlorsäure im Verhältnis 9:1. Die Reaktionstemperaturen für die Spaltung liegen zweckmäßig zwischen etwa 0 und etwa 50°, vorzugsweise arbeitet man zwischen 15 und 30° (Raumtemperatur).

Die Gruppen BOC, OBut und Mtr können z. B. bevorzugt mit TFA in Dichlormethan oder mit etwa 3 bis 5n HCl in Dioxan bei 15-30° abgespalten werden, die FMOC-Gruppe mit einer etwa 5- bis 50 %igen Lösung von Dimethylamin, Diethylamin oder Piperidin in DMF bei 15-30°.

Hydrogenolytisch entfernbare Schutzgruppen (z. B. CBZ, Benzyl oder die Freisetzung der Amidinogruppe aus ihrem Oxadiazolderivat)) können z. B. durch Behandeln mit Wasserstoff in Gegenwart eines Katalysators (z. B. eines Edelmetallkatalysators wie Palladium, zweckmäßig auf einem Träger wie Kohle) abgespalten werden. Als Lösungsmittel eignen sich dabei die oben angegebenen, insbesondere z. B. Alkohole wie Methanol oder Etha-

nol oder Amide wie DMF. Die Hydrogenolyse wird in der Regel bei Temperaturen zwischen etwa 0 und 100° und Drucken zwischen etwa 1 und 200 bar, bevorzugt bei 20-30° und 1-10 bar durchgeführt. Eine Hydrogenolyse der CBZ-Gruppe gelingt z. B. gut an 5 bis 10 %igem Pd/C in Methanol oder mit Ammomiumformiat (anstelle von Wasserstoff) an Pd/C in Methanol/DMF bei 20-30°.

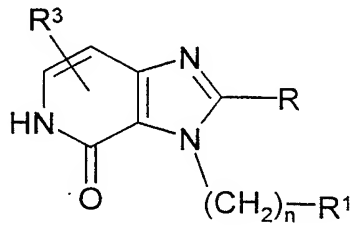
Als inerte Lösungsmittel eignen sich z.B. Kohlenwasserstoffe wie Hexan, Petrolether, Benzol, Toluol oder Xylol; chlorierte Kohlenwasserstoffe wie Trichlorethylen, 1,2-Dichlorethan,Tetrachlorkohlenstoff, Trifluormethylbenzol, Chloroform oder Dichlormethan; Alkohole wie Methanol, Ethanol, Isopropanol, n-Propanol, n-Butanol oder tert.-Butanol; Ether wie Diethylether, Diisopropylether, Tetrahydrofuran (THF) oder Dioxan; Glykolether wie Ethylenglykolmonomethyl- oder -monoethylether (Methylglykol oder Ethylglykol), Ethylenglykoldimethylether (Diglyme); Ketone wie Aceton oder Butanon; Amide wie Acetamid, Dimethylacetamid, N-Methylpyrrolidon (NMP) oder Dimethylformamid (DMF); Nitrile wie Acetonitril; Sulfoxide wie Dimethylsulfoxid (DMSO); Schwefelkohlenstoff; Carbonsäuren wie Ameisensäure oder Essigsäure; Nitroverbindungen wie Nitromethan oder Nitrobenzol; Ester wie Ethylacetat oder Gemische der genannten Lösungsmittel.

Die Biphenyl-SO_2NH_2-Gruppe wird vorzugsweise in Form ihres tert.-Butylderivates eingesetzt. Die Abspaltung der tert.-Butylgruppe erfolgt z.B. mit TFA mit oder ohne Zusatz eines inerten Lösungsmittels, vorzugsweise unter Zusatz einer geringen Menge an Anisol (1 Vol %).

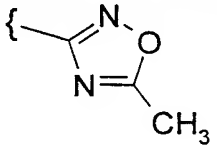
Die Umwandlung einer Cyangruppe in eine Amidinogruppe erfolgt durch Umsetzung mit z.B. Hydroxylamin und anschließender Reduktion des N-Hydroxyamidins mit Wasserstoff in Anwesenheit eines Katalysators wie z.B. Pd/C.
Zur Herstellung eines Amidins der Formel I (z.B. Ar = einfach durch $C(=NH)-NH_2$ substituiertes Phenyl) kann man an ein Nitril auch Ammoniak anlagern. Die Anlagerung erfolgt bevorzugt mehrstufig, indem man in an sich bekannter Weise a) das Nitril mit H_2S in ein Thioamid umwandelt, das mit einem Alkylierungsmittel, z.B. CH_3I, in den entsprechenden S-Alkyl-

imidothioester übergeführt wird, welcher seinerseits mit NH_3 zum Amidin reagiert, b) das Nitril mit einem Alkohol, z.B. Ethanol in Gegenwart von HCl in den entsprechenden Imidoester umwandelt und diesen mit Ammoniak behandelt, oder c) das Nitril mit Lithium-bis-(trimethylsilyl)-amid umsetzt und das Produkt anschließend hydrolysiert.

Die Einführung der Reste R^2 bzw. $-(CH_2)_n-R^1$ in das Dihydro-imidazo[4,5-*c*]-pyridin-4-on-System erfolgt nach üblichen Alkylierungsmethoden.
So kann man z.B. eine Verbindung der Formel II



II

worin R die in Anspruch 1 angegebene Bedeutung hat und R^1 und R^3 jeweils einen solchen Rest bedeuten, der nicht alkylierbar ist, wie z.B. für R^1 einen durch  substituierten Phenyl- oder Naphthylrest,

mit einer Verbindung der Formel III

$$R^2\text{-}(CH_2)_p\text{-}L \qquad \text{III}$$

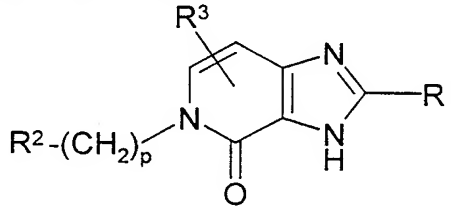
worin L Cl, Br, I oder eine freie oder reaktionsfähig funktionell abgewandelte OH-Gruppe bedeutet, und p 1 bedeutet,
umsetzen und erhält durch dieses Verfahren Verbindungen der Formel (IA).

L bedeutet vorzugsweise Cl, Br, I oder eine reaktionsfähig abgewandelte OH-Gruppe wie z.B. ein aktivierter Ester, ein Imidazolid oder Alkylsulfonyloxy mit 1-6 C-Atomen (bevorzugt Methylsulfonyloxy) oder Arylsulfonyloxy mit 6-10 C-Atomen (bevorzugt Phenyl- oder p-Tolylsulfonyloxy).

Als Lösungsmittel eignen sich die oben angeführten. Die Reaktion erfolgt in Gegenwart eines säurebindenden Mittels vorzugsweise eines Alkali- oder Erdalkalimetall-hydroxids, -carbonats oder -bicarbonats oder eines anderen Salzes einer schwachen Säure der Alkali- oder Erdalkalimetalle, vorzugsweise des Kaliums, Natriums, Calciums oder Cäsiums. Auch der Zusatz einer organischen Base wie Triethylamin, Dimethylanilin, Pyridin oder Chinolin kann günstig sein. Die Reaktionszeit liegt je nach den angewendeten Bedingungen zwischen einigen Minuten und 14 Tagen, die Reaktionstemperatur zwischen etwa 0° und 150°, normalerweise zwischen 20° und 130°.

In Verbindungen mit p = 0, wird R^2 über ein Boronsäurederivat eingeführt.

Analog kann auch zuerst R^2-$(CH_2)_p$-, worin p = 1 ist, in das Dihydro-imidazo[4,5-*c*]-pyridin-4-on-System eingeführt werden und anschließend eine Verbindung der Formel IV



IV

worin p = 1 ist und R die in Anspruch 1 angegebene Bedeutung hat und R^2 und R^3 jeweils einen solchen Rest bedeuten, der nicht alkylierbar ist, mit einer Verbindung der Formel V

$$R^1\text{-}(CH_2)_n\text{-}L \qquad V$$

umgesetzt werden.
In den Verbindungen der Formel V bedeutet R^1 einen nicht alkylierbaren Rest, wie z.B. einen durch 5-Methyl-[1,2,4]oxadiazol-3-yl substituierten Phenylrest und L hat die Bedeutung wie in den Verbindungen der Formel III. Man erhält durch dieses Verfahren Verbindungen der Formel (IA) und/oder (IB).

Es ist ferner möglich, eine Verbindung der Formel I in eine andere Verbindung der Formel I umzuwandeln, indem man einen oder mehrere Rest(e) R, R^1, R^2 und/oder R^3 in einen oder mehrere Rest(e) R, R^1, R^2,

und/oder R^3 umwandelt, z.B. indem man eine Aminogruppe acyliert oder Nitrogruppen (beispielsweise durch Hydrierung an Raney-Nickel oder Pd-Kohle in einem inerten Lösungsmittel wie Methanol oder Ethanol) zu Aminogruppen reduziert.

Ester können z.B. mit Essigsäure oder mit NaOH oder KOH in Wasser, Wasser-THF oder Wasser-Dioxan bei Temperaturen zwischen 0 und 100° verseift werden.

Ferner kann man freie Aminogruppen in üblicher Weise mit einem Säurechlorid oder -anhydrid acylieren oder mit einem unsubstituierten oder substituierten Alkylhalogenid alkylieren, zweckmäßig in einem inerten Lösungsmittel wie Dichlormethan oder THF und /oder in Gegenwart einer Base wie Triethylamin oder Pyridin bei Temperaturen zwischen -60 und +30°.

Eine Base der Formel I kann mit einer Säure in das zugehörige Säureadditionssalz übergeführt werden, beispielsweise durch Umsetzung äquivalenter Mengen der Base und der Säure in einem inerten Lösungsmittel wie Ethanol und anschließendes Eindampfen. Für diese Umsetzung kommen insbesondere Säuren in Frage, die physiologisch unbedenkliche Salze liefern. So können anorganische Säuren verwendet werden, z.B. Schwefelsäure, Salpetersäure, Halogenwasserstoffsäuren wie Chlorwasserstoffsäure oder Bromwasserstoffsäure, Phosphorsäuren wie Orthophosphorsäure, Sulfaminsäure, ferner organische Säuren, insbesondere aliphatische, alicyclische, araliphatische, aromatische oder heterocyclische ein- oder mehrbasige Carbon-, Sulfon- oder Schwefelsäuren, z.B. Ameisensäure, Essigsäure, Propionsäure, Pivalinsäure, Diethylessigsäure, Malonsäure, Bernsteinsäure, Pimelinsäure, Fumarsäure, Maleinsäure, Milchsäure, Weinsäure, Äpfelsäure, Citronensäure, Gluconsäure, Ascorbinsäure, Nicotinsäure, Isonicotinsäure, Methan- oder Ethansulfonsäure, Ethandisulfonsäure, 2-Hydroxyethansulfonsäure, Benzolsulfonsäure, p-Toluolsulfonsäure, Naphthalin-mono- und -disulfonsäuren, Laurylschwefelsäure. Salze mit physiologisch nicht unbedenklichen Säuren, z.B. Pikrate, können zur Isolierung und /oder Aufreinigung der Verbindungen der Formel I verwendet werden.

Andererseits können Verbindungen der Formel I mit Basen (z.B. Natrium- oder Kaliumhydroxid oder -carbonat) in die entsprechenden Metall-, insbesondere Alkalimetall- oder Erdalkalimetall-, oder in die entsprechenden Ammoniumsalze umgewandelt werden.
Auch physiologisch unbedenkliche organische Basen, wie z.B. Ethanolamin können verwendet werden.

Erfindungsgemäße Verbindungen der Formel I können aufgrund ihrer Molekülstruktur chiral sein und können dementsprechend in verschiedenen enantiomeren Formen auftreten. Sie können daher in racemischer oder in optisch aktiver Form vorliegen.

Da sich die pharmazeutische Wirksamkeit der Racemate bzw. der Stereoisomeren der erfindungsgemäßen Verbindungen unterscheiden kann, kann es wünschenswert sein, die Enantiomere zu verwenden. In diesen Fällen kann das Endprodukt oder aber bereits die Zwischenprodukte in enantiomere Verbindungen, durch dem Fachmann bekannte chemische oder physikalische Maßnahmen, aufgetrennt oder bereits als solche bei der Synthese eingesetzt werden.

Im Falle racemischer Amine werden aus dem Gemisch durch Umsetzung mit einem optisch aktiven Trennmittel Diastereomere gebildet. Als Trennmittel eignen sich z.B. optisch aktiven Säuren, wie die R- und S-Formen von Weinsäure, Diacetylweinsäure, Dibenzoylweinsäure, Mandelsäure, Äpfelsäure, Milchsäure, geeignet N-geschützte Aminosäuren (z.B. N-Benzoylprolin oder N-Benzolsulfonylprolin) oder die verschiedenen optisch aktiven Camphersulfonsäuren. Vorteilhaft ist auch eine chromatographische Enantiomerentrennung mit Hilfe eines optisch aktiven Trennmittels (z.B. Dinitrobenzoylphenylglycin, Cellulosetriacetat oder andere Derivate von Kohlenhydraten oder auf Kieselgel fixierte chiral derivatisierte Methacrylatpolymere). Als Laufmittel eignen sich hierfür wäßrige oder alkoholische Lösungsmittelgemische wie z.B. Hexan/Isopropanol/ Acetonitril z.B. im Verhältnis 82:15:3.

Gegenstand der Erfindung ist ferner die Verwendung der Verbindungen der Formel I und/oder ihrer physiologisch unbedenklichen Salze zur Her-

stellung pharmazeutischer Zubereitungen, insbesondere auf nicht-chemischem Wege. Hierbei können sie zusammen mit mindestens einem festen, flüssigen und/oder halbflüssigen Träger- oder Hilfsstoff und gegebenenfalls in Kombination mit einem oder mehreren weiteren Wirkstoffen in eine geeignete Dosierungsform gebracht werden.

Gegenstand der Erfindung sind ferner pharmazeutische Zubereitungen, enthaltend mindestens eine Verbindung der Formel I und/oder eines ihrer physiologisch unbedenklichen Salze.

Diese Zubereitungen können als Arzneimittel in der Human- oder Veterinärmedizin verwendet werden. Als Trägerstoffe kommen organische oder anorganische Substanzen in Frage, die sich für die enterale (z.B. orale), parenterale oder topische Applikation eignen und mit den neuen Verbindungen nicht reagieren, beispielsweise Wasser, pflanzliche Öle, Benzylalkohole, Alkylenglykole, Polyethylenglykole, Glycerintriacetat, Gelatine, Kohlehydrate wie Lactose oder Stärke, Magnesiumstearat, Talk, Vaseline. Zur oralen Anwendung dienen insbesondere Tabletten, Pillen, Dragees, Kapseln, Pulver, Granulate, Sirupe, Säfte oder Tropfen, zur rektalen Anwendung Suppositorien, zur parenteralen Anwendung Lösungen, vorzugsweise ölige oder wässrige Lösungen, ferner Suspensionen, Emulsionen oder Implantate, für die topische Anwendung Salben, Cremes oder Puder. Die neuen Verbindungen können auch lyophilisiert und die erhaltenen Lyophilisate z.B. zur Herstellung von Injektionspräparaten verwendet werden. Die angegebenen Zubereitungen können sterilisiert sein und/oder Hilfsstoffe wie Gleit-, Konservierungs-, Stabilisierungs- und/oder Netzmittel, Emulgatoren, Salze zur Beeinflussung des osmotischen Druckes, Puffersubstanzen, Farb-, Geschmacks- und /oder mehrere weitere Wirkstoffe enthalten, z.B. ein oder mehrere Vitamine.

Die Verbindungen der Formel I und ihre physiologisch unbedenklichen Salze können bei der Bekämpfung und Verhütung von thromboembolischen Erkrankungen wie Thrombose, myocardialem Infarkt, Arteriosklerose, Entzündungen, Apoplexie, Angina pectoris, Restenose nach Angioplastie und Claudicatio intermittens verwendet werden.

Dabei werden die erfindungsgemäßen Substanzen in der Regel vorzugsweise in Dosierungen zwischen etwa 1 und 500 mg, insbesondere zwischen 5 und 100 mg pro Dosierungseinheit verabreicht. Die tägliche Dosierung liegt vorzugsweise zwischen etwa 0,02 und 10 mg/kg Körpergewicht. Die spezielle Dosis für jeden Patienten hängt jedoch von den verschiedensten Faktoren ab, beispielsweise von der Wirksamkeit der eingesetzten speziellen Verbindung, vom Alter, Körpergewicht, allgemeinen Gesundheitszustand, Geschlecht, von der Kost, vom Verabreichungszeitpunkt und -weg, von der Ausscheidungsgeschwindigkeit, Arzneistoffkombination und Schwere der jeweiligen Erkrankung, welcher die Therapie gilt. Die orale Applikation ist bevorzugt.

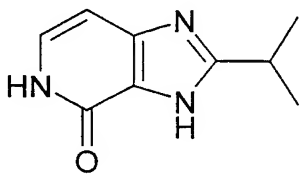
Vor- und nachstehend sind alle Temperaturen in °C angegeben. In den nachfolgenden Beispielen bedeutet "übliche Aufarbeitung": Man gibt, falls erforderlich, Wasser hinzu, stellt, falls erforderlich, je nach Konstitution des Endprodukts auf pH-Werte zwischen 2 und 10 ein, extrahiert mit Ethylacetat oder Dichlormethan, trennt ab, trocknet die organische Phase über Natriumsulfat, dampft ein und reinigt durch Chromatographie an Kieselgel und /oder durch Kristallisation. Rf-Werte an Kieselgel; Laufmittel: Ethylacetat/Methanol 9:1.

Massenspektrometrie (MS): EI (Elektronenstoß-Ionisation) M^+

FAB (Fast Atom Bombardment) $(M+H)^+$

Beispiel 1

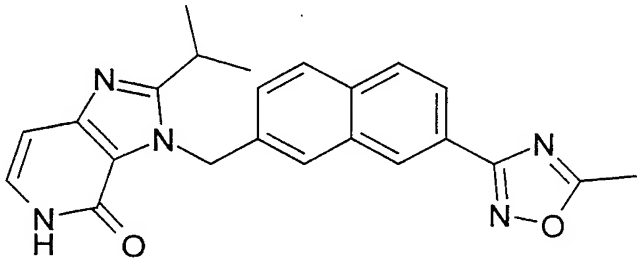
Zu 50,0 g 3,4-Diamino-2-chlorpyridin werden 140 mL Isobuttersäure und 250 mL rauchende Salzsäure gegeben. Das Reaktionsgemisch wird 7 Tage unter Rückfluß erhitzt. Man gießt in Eiswasser, trennt den ausgefallenen Niederschlag ab und erhält 2-Isopropyl-3,5-dihydro-imidazo[4,5-*c*]-pyridin-4-on ("AB"), F. 310-311° (Zersetzung), EI 177



"AB" .

In der Mutterlauge befindet sich ein Gemisch aus "AB" und 4-Chloro-2-isopropyl-3*H*-imidazo[4,5-*c*]-pyridin.

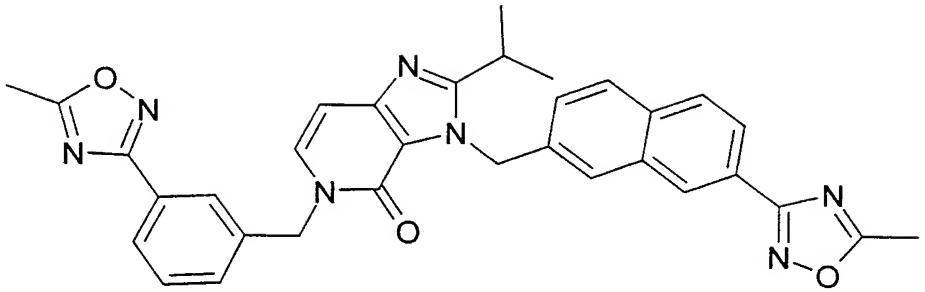
Eine Lösung von 0,877 g "AB" und 0,691 g Kaliumcarbonat in 30 mL DMF wird 30 Minuten bei Raumtemperatur gerührt. Man fügt 1,5 g 3-(7-Bromomethyl-naphthalin-2-yl)-5-methyl-[1,2,4]oxadiazol (F. 149-150°) hinzu und rührt 16 Stunden nach und arbeitet wie üblich auf. Nach Chromatographie über Kieselgel erhält man neben den beiden regioisomeren Dialkylierungsprodukten die Verbindung 2-Isopropyl-3-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-yl-methyl]-5*H*-imidazo[4,5-*c*]pyridin-4-on ("BB"), F. 214-215°, EI 399



"BB" .

Ein alternatives Verfahren führt wie folgt zu "BB" (analog Mederski et al., J. Med. Chem. **1994**, 1632 ff):
Umsetzung von 3,4-Diamino-2-chlorpyridin mit Isobutyranhydrid zu N-(4-Amino-2-chloro-pyridin-3-yl)-isobutyramid. Die anschließende Umsetzung mit 3-(7-Bromomethyl-naphthalin-2-yl)-5-methyl-[1,2,4]oxadiazol führt zu einem Gemisch aus 4-Chloro-2-isopropyl-3-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-yl-methyl]-3*H*-imidazo[4,5-*c*]pyridin und N-(4-Amino-2-chloro-pyridin-3-yl)-N-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-yl-methyl]-isobutyramid. Beide Verbindungen werden zu "BB" umgesetzt.

Zu einer Lösung von 0,2 g "BB" in 10 mL DMF gibt man 62 mg Kaliumtertiärbutylat und rührt 30 Minuten. Anschließend fügt man 0,140 g 3-(3-Bromomethylphenyl)-5-methyl-[1,2,4]oxadiazol dazu und rührt weitere 2 Stunden nach. Nach üblicher Aufarbeitung erhält man die Verbindung
2-Isopropyl-3-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-yl-methyl]-5-[3-(5-methyl-[1,2,4]oxadiazol-3-yl)-benzyl]-3,5-dihydro-imidazo[4,5-*c*]-pyridin-4-on ("BC1"), F. 108-109°, EI 571



"BC1".

Analog erhält man durch Umsetzung von "BB" mit

3-(7-Bromomethyl-naphthalin-2-yl)-5-methyl-[1,2,4]oxadiazol,
3-(4-Bromomethylphenyl)-5-methyl-[1,2,4]oxadiazol,
3-(2-Bromomethylphenyl)-5-methyl-[1,2,4]oxadiazol,
Benzylbromid,
3-Dimethylaminocarbonyl-benzylbromid,
3'-(N-tert.-Butyl-sulfonamido)-biphenyl-3-yl-methylbromid,

die nachstehenden Verbindungen

2-Isopropyl-3-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-yl-methyl]-5-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-ylmethyl]-3,5-dihydro-imidazo-[4,5-*c*]pyridin-4-on ("BC2"), F. 201-202°;

2-Isopropyl-3-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-yl-methyl]-5-[4-(5-methyl-[1,2,4]oxadiazol-3-yl)-benzyl]-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on ("BC3"), F. 172-173°;

2-Isopropyl-3-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-yl-methyl]-5-[2-(5-methyl-[1,2,4]oxadiazol-3-yl)-benzyl]-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on ("BC4"), F. 149-150°;

2-Isopropyl-3-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-yl-methyl]-5-benzyl-5*H*-imidazo[4,5-*c*]pyridin-4-on ("BC5"), F. 112-113°;

2-Isopropyl-3-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-yl-methyl]-5-(3-dimethylaminocarbonyl-benzyl)-5*H*-imidazo[4,5-*c*]pyridin-4-on ("BC6"),

2-Isopropyl-3-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-yl-methyl]-5-[3'-(N-tert.-butyl-sulfonamido)-biphenyl-3-ylmethyl]-5*H*-imidazo[4,5-*c*]pyridin-4-on ("BC7"), FAB 639.

Beispiel 2

Alternatives Verfahren zur Herstellung von "BC1"

Durch Umsetzung von 3,4-Diamino-2-chlorpyridin mit Isobuttersäure und anschließend mit Di-(tert.-butyloxy)-anhydrid (analog WO 97/21437, S. 44-45) erhält man ein Gemisch von 2-Isopropyl-3-tert.-butyloxycarbonyl-5*H*-imidazo[4,5-*c*]pyridin-4-on und 2-Isopropyl-1-tert.-butyloxycarbonyl-5*H*-imidazo[4,5-*c*]pyridin-4-on.
Das Gemisch der beiden Verbindungen wird analog Beispiel 1 mit 3-(3-Bromomethylphenyl)-5-methyl-[1,2,4]oxadiazol umgesetzt und man erhält ein Gemisch der beiden Verbindungen

2-Isopropyl-3-tert.-butyloxycarbonyl-5-[3-(5-methyl-[1,2,4]oxadiazol-3-yl)-benzyl]-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on und

2-Isopropyl-1-tert.-butyloxycarbonyl-5-[3-(5-methyl-[1,2,4]oxadiazol-3-yl)-benzyl]-1,5-dihydro-imidazo[4,5-*c*]pyridin-4-on.

Nach Abspaltung der BOC-Schutzgruppen mit TFA in Dioxan und üblicher Aufarbeitung wird mit 3-(7-Bromomethyl-naphthalin-2-yl)-5-methyl-[1,2,4]-oxadiazol analog Beispiel 1 umgesetzt. Nach üblicher Aufarbeitung erhält man ein Gemisch regioisomerer Produkte, aus dem "BC1" durch Chromatographie abgetrennt wird.

Beispiel 3

Eine Lösung von 0,2 g "BC1" in 20 mL Methanol wird mit 100 mg Raney-Nickel und einem Tropfen Essigsäure versetzt und 8 Stunden bei Raumtemperatur hydriert. Der Katalysator wird abfiltriert, das Lösungsmittel entfernt und man erhält die Verbindung

2-Isopropyl-3-(7-amidino-naphth-2-yl-methyl)-5-(3-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on, F. > 300° (Zersetzung), FAB 492.

Analog erhält man aus "BC2", "BC3", "BC4", "BC5", "BC6" und "BC7" die nachstehenden Verbindungen

2-Isopropyl-3-(7-amidino-naphth-2-yl-methyl)-5-(7-amidino-naphth-2-ylmethyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on, F. > 300°, EI 166;

2-Isopropyl-3-(7-amidino-naphth-2-yl-methyl)-5-(4-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on, F. 208-209° (Zersetzung), FAB 492;

2-Isopropyl-3-(7-amidino-naphth-2-yl-methyl)-5-(2-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on, F. > 300°, FAB 492;

2-Isopropyl-3-[7-amidino-naphth-2-yl-methyl]-5-benzyl-5*H*-imidazo[4,5-*c*]pyridin-4-on, F. 206-207 (Zersetzung), FAB 450;

2-Isopropyl-3-[7-amidino-naphth-2-yl-methyl]-5-(3-dimethylamino-carbonyl-benzyl)-5*H*-imidazo[4,5-*c*]pyridin-4-on,

2-Isopropyl-3-[7-amidino-naphth-2-yl-methyl]-5-[3'-(N-tert.-butyl-sulfonamido)-biphenyl-3-ylmethyl]-5*H*-imidazo[4,5-*c*]pyridin-4-on ("DF").

Analog erhält man 3-(7-amidino-naphth-2-yl-methyl)-5-(3-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on, F. > 300°, FAB 450.

Beispiel 4

Analog Beispiel 1 erhält man durch Umsetzung von "AB" mit 3-(3-Bromomethylphenyl)-5-methyl-[1,2,4]oxadiazol die Verbindung 2-Isopropyl-3-[3-(5-methyl-[1,2,4]oxadiazol-3-yl)-benzyl]-5*H*-imidazo[4,5-*c*]-pyridin-4-on ("CA").

Durch Umsetzung von "CA" mit

3-(3-Bromomethylphenyl)-5-methyl-[1,2,4]oxadiazol,
3-(7-Bromomethyl-naphthalin-2-yl)-5-methyl-[1,2,4]oxadiazol,

3-(4-Bromomethylphenyl)-5-methyl-[1,2,4]oxadiazol,
3-(2-Bromomethylphenyl)-5-methyl-[1,2,4]oxadiazol,

erhält man die dialkylierten Imidazoderivate, die durch Hydrierung analog Beispiel 3 in die nachstehenden Verbindungen überführt werden

2-Isopropyl-3-(3-amidino-benzyl)-5-(3-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Isopropyl-3-(3-amidino-benzyl)-5-(7-amidino-naphth-2-ylmethyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Isopropyl-3-(3-amidino-benzyl)-5-(4-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Isopropyl-3-(3-amidino-benzyl)-5-(2-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on.

Beispiel 5

Durch Umsetzung von 3,4-Diamino-2-chlorpyridin analog Beispiel 1 mit den nachstehenden Carbonsäuren

Propionsäure,
Cyclopropylcarbonsäure,

anschließender Alkylierung der entstehenden Imidazoderivate analog den Beispielen 1 und 4 und Hydrierung analog Beispiel 3 erhält man die nachstehenden Verbindungen

2-Ethyl-3-(7-amidino-naphth-2-yl-methyl)-5-(3-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on, F. 145°, FAB 478;

2-Ethyl-3-(7-amidino-naphth-2-yl-methyl)-5-(7-amidino-naphth-2-ylmethyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Ethyl-3-(7-amidino-naphth-2-yl-methyl)-5-(4-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Ethyl-3-(7-amidino-naphth-2-yl-methyl)-5-(2-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on.

2-Ethyl-3-(3-amidino-benzyl)-5-(3-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Ethyl-3-(3-amidino-benzyl)-5-(7-amidino-naphth-2-ylmethyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Ethyl-3-(3-amidino-benzyl)-5-(4-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Ethyl-3-(3-amidino-benzyl)-5-(2-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Cyclopropyl-3-(7-amidino-naphth-2-yl-methyl)-5-(3-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Cyclopropyl-3-(7-amidino-naphth-2-yl-methyl)-5-(7-amidino-naphth-2-ylmethyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Cyclopropyl-3-(7-amidino-naphth-2-yl-methyl)-5-(4-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Cyclopropyl-3-(7-amidino-naphth-2-yl-methyl)-5-(2-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on.

2-Cyclopropyl-3-(3-amidino-benzyl)-5-(3-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Cyclopropyl-3-(3-amidino-benzyl)-5-(7-amidino-naphth-2-ylmethyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Cyclopropyl-3-(3-amidino-benzyl)-5-(4-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Cyclopropyl-3-(3-amidino-benzyl)-5-(2-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on.

Analog erhält man die Verbindungen

2-Isobutyl-3-(7-amidino-naphth-2-yl-methyl)-5-(3-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on, F. 69-70°, FAB 506;

2-Methyl-3-(7-amidino-naphth-2-yl-methyl)-5-(3-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on, F. 171-172°, FAB 464;

2-Butyl-3-(7-amidino-naphth-2-yl-methyl)-5-(3-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on, F. 190-191°, FAB 506.

Beispiel 6

Durch Umsetzung von 3,4-Diamino-2-chlor-5-methoxycarbonylpyridin (F. 181-184°) analog Beispiel 1 mit Isobuttersäure erhält man 2-Isopropyl-3,5-dihydro-7-carboxy-imidazo[4,5-*c*]pyridin-4-on. Die Carbonsäure wird nach üblichen Methoden zu 2-Isopropyl-3,5-dihydro-7-methoxycarbonyl-imidazo-[4,5-*c*]pyridin-4-on umgesetzt und anschließend analog den Beispielen 1 und 4 alkyliert und analog Beispiel 3 hydriert. Dabei werden nachstehende Carbonsäurederivate erhalten

7-Carboxy-2-isopropyl-3-(7-amidino-naphth-2-yl-methyl)-5-(3-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

7-Carboxy-2-isopropyl-3-(7-amidino-naphth-2-yl-methyl)-5-(7-amidino-naphth-2-ylmethyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

7-Carboxy-2-isopropyl-3-(7-amidino-naphth-2-yl-methyl)-5-(4-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

7-Carboxy-2-isopropyl-3-(7-amidino-naphth-2-yl-methyl)-5-(2-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on.

7-Carboxy-2-isopropyl-3-(3-amidino-benzyl)-5-(3-amidino-benzyl)-3,5-dihydro- imidazo[4,5-*c*]pyridin-4-on,

7-Carboxy-2-isopropyl-3-(3-amidino-benzyl)-5-(7-amidino-naphth-2-ylmethyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

7-Carboxy-2-isopropyl-3-(3-amidino-benzyl)-5-(4-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

7-Carboxy-2-isopropyl-3-(3-amidino-benzyl)-5-(2-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on.

Beispiel 7

Durch Umsetzung von 3,4-Diamino-2-chlor-5-brompyridin (F. 206-208°) analog Beispiel 1 mit Isobuttersäure erhält man 2-Isopropyl-3,5-dihydro-7-brom-imidazo[4,5-*c*]pyridin-4-on. Dieses wird anschließend analog den Beispielen 1 und 4 alkyliert und analog Beispiel 3 hydriert. Dabei werden nachstehende Verbindungen erhalten

7-Brom-2-isopropyl-3-(7-amidino-naphth-2-yl-methyl)-5-(3-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

7-Brom-2-isopropyl-3-(7-amidino-naphth-2-yl-methyl)-5-(7-amidino-naphth-2-ylmethyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

7-Brom-2-isopropyl-3-(7-amidino-naphth-2-yl-methyl)-5-(4-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

7-Brom-2-isopropyl-3-(7-amidino-naphth-2-yl-methyl)-5-(2-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on.

7-Brom-2-isopropyl-3-(3-amidino-benzyl)-5-(3-amidino-benzyl)-3,5-dihydro- imidazo[4,5-*c*]pyridin-4-on,

7-Brom-2-isopropyl-3-(3-amidino-benzyl)-5-(7-amidino-naphth-2-ylmethyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

7-Brom-2-isopropyl-3-(3-amidino-benzyl)-5-(4-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

7-Brom-2-isopropyl-3-(3-amidino-benzyl)-5-(2-amidino-benzyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on.

Beispiel 8

Durch Umsetzung von 2-Isopropyl-3,5-dihydro-7-brom-imidazo[4,5-*c*]-pyridin-4-on nach üblichen Methoden mit CuCN in DMF (Ellefson et al., J. Med. Chem. **1976**, 19) erhält man 2-Isopropyl-3,5-dihydro-7-cyan-imidazo[4,5-*c*]pyridin-4-on.
Dieses wird anschließend verseift und analog den Beispielen 1 und 4 alkyliert und analog Beispiel 3 hydriert. Dabei werden die unter Beispiel 6 aufgeführten Carbonsäurederivate erhalten.

Beispiel 9

Analog Beispiel 1 erhält man durch Umsetzung von "AB" mit 3-(3-Bromomethyl-biphenyl-3'-yl)-5-methyl-[1,2,4]oxadiazol, üblicher Aufarbeitung und Chromatographie die Verbindung 2-Isopropyl-3-[3'-(5-methyl-[1,2,4]oxadiazol-3-yl)-biphenyl-3-yl-methyl]-5*H*-imidazo[4,5-*c*]pyridin-4-on ("CD").

Durch Umsetzung von "CD" mit

3-(3-Bromomethylphenyl)-5-methyl-[1,2,4]oxadiazol,
3-(3-Bromomethyl-biphenyl-3'-yl)-5-methyl-[1,2,4]oxadiazol,

und anschließender Hydrierung erhält man die nachstehenden Verbindungen

2-Isopropyl-3-[3'-amidino-biphenyl-3-yl-methyl]-5-(3-amidinobenzyl)-5*H*-imidazo[4,5-*c*]pyridin-4-on und

2-Isopropyl-3-[3'-amidino-biphenyl-3-yl-methyl]-5-[3'-amidino-biphenyl-3-yl-methyl]-5*H*-imidazo[4,5-*c*]pyridin-4-on.

Analog erhält man die Verbindung

2-Isopropyl-3-[4'-amidino-biphenyl-3-yl-methyl]-5-benzyl-5*H*-imidazo[4,5-*c*]-pyridin-4-on, F. > 300°; EI 475.

Beispiel 10

Aus "DF" erhält man nach üblichen Methoden durch Abspaltung der tert.-Butylgruppe in TFA die Verbindung

2-Isopropyl-3-[7-amidino-naphth-2-yl-methyl]-3-(3'-sulfonamido-biphenyl-3-ylmethyl)-5*H*-imidazo[4,5-*c*]pyridin-4-on.

Analog erhält man die Verbindungen

2-Isopropyl-5-(3-amidino-benzyl)-3-(3'-sulfonamido-biphenyl-3-ylmethyl)-5*H*-imidazo[4,5-*c*]pyridin-4-on und

2-Isopropyl-5-(4-amidino-benzyl)-3-(3'-sulfonamido-biphenyl-3-ylmethyl)-5*H*-imidazo[4,5-*c*]pyridin-4-on.

Beispiel 11

Durch Umsetzung von 2-Isopropyl-3-[3-(5-methyl-[1,2,4]oxadiazol-3-yl)-benzyl]-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on mit 3-Cyan-phenylboronsäure unter Kupferacetatkatalyse in Dichlormethan erhält man 2-Isopropyl-3-[3-(5-methyl-[1,2,4]oxadiazol-3-yl)-benzyl]-5-(3-cyanphenyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on. Durch anschließende Umsetzung in Ethanol $NaHCO_3$ und danach mit Hydroxylammoniumchlorid erhält man 2-Isopropyl-3-[3-(5-methyl-[1,2,4]oxadiazol-3-yl)-benzyl]-5-(3-N-hydroxy-amidinophenyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on.

Nach Hydrierung analog Beispiel 3 erhält man 2-Isopropyl-3-[3-amidino-benzyl]-5-(3-amidinophenyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on, FAB 428.

Analog erhält man nachstehende Verbindungen

2-Isopropyl-3-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-ylmethyl]-5-(3-cyanphenyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-tert.-Butyl-3-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-ylmethyl]-5-(3-cyanphenyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Butyl-3-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-ylmethyl]-5-(3-cyanphenyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Isobutyl-3-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-ylmethyl]-5-(3-cyanphenyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Isopropyl-3-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-ylmethyl]-5-(4-cyanphenyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-tert.-Butyl-3-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-ylmethyl]-5-(4-cyanphenyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Butyl-3-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-ylmethyl]-5-(4-cyanphenyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on,

2-Isobutyl-3-[7-(5-methyl-[1,2,4]oxadiazol-3-yl)-naphth-2-ylmethyl]-5-(4-cyanphenyl)-3,5-dihydro-imidazo[4,5-*c*]pyridin-4-on.

Durch Umsetzung mit Hydroxylammoniumchlorid und nachfolgender Hydrierung erhält man daraus die Diamidinoverbindungen.

Die nachfolgenden Beispiele betreffen pharmazeutische Zubereitungen:

Beispiel A: Injektionsgläser

Eine Lösung von 100 g eines Wirkstoffes der Formel I und 5 g Dinatriumhydrogenphosphat wird in 3 l zweifach destilliertem Wasser mit 2 n Salzsäure auf pH 6,5 eingestellt, steril filtriert, in Injektionsgläser abgefüllt, unter sterilen Bedingungen lyophilisiert und steril verschlossen. Jedes Injektionsglas enthält 5 mg Wirkstoff.

Beispiel B: Suppositorien

Man schmilzt ein Gemisch von 20 g eines Wirkstoffes der Formel I mit 100 g Sojalecithin und 1400 g Kakaobutter, gießt in Formen und läßt erkalten. Jedes Suppositorium enthält 20 mg Wirkstoff.

Beispiel C: Lösung

Man bereitet eine Lösung aus 1 g eines Wirkstoffes der Formel I, 9,38 g $NaH_2PO_4 \cdot 2\ H_2O$, 28,48 g $Na_2HPO_4 \cdot 12\ H_2O$ und 0,1 g Benzalkoniumchlorid in 940 ml zweifach destilliertem Wasser. Man stellt auf pH 6,8 ein, füllt auf 1 l auf und sterilisiert durch Bestrahlung. Diese Lösung kann in Form von Augentropfen verwendet werden.

Beispiel D: Salbe

Man mischt 500 mg eines Wirkstoffes der Formel I mit 99,5 g Vaseline unter aseptischen Bedingungen.

Beispiel E: Tabletten

Ein Gemisch von 1 kg Wirkstoff der Formel I, 4 kg Lactose, 1,2 kg Kartoffelstärke, 0,2 kg Talk und 0,1 kg Magnesiumstearat wird in üblicher Weise zu Tabletten verpreßt, derart, daß jede Tablette 10 mg Wirkstoff enthält.

Beispiel F: Dragees

Analog Beispiel E werden Tabletten gepreßt, die anschließend in üblicher Weise mit einem Überzug aus Saccharose, Kartoffelstärke, Talk, Tragant und Farbstoff überzogen werden.

Beispiel G: Kapseln

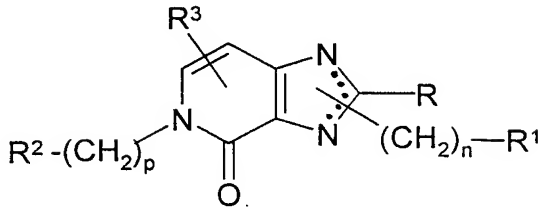
2 kg Wirkstoff der Formel I werden in üblicher Weise in Hartgelatinekapseln gefüllt, so daß jede Kapsel 20 mg des Wirkstoffs enthält.

Beispiel H: Ampullen

Eine Lösung von 1 kg Wirkstoff der Formel I in 60 l zweifach destilliertem Wasser wird steril filtriert, in Ampullen abgefüllt, unter sterilen Bedingungen lyophilisiert und steril verschlossen. Jede Ampulle enthält 10 mg Wirkstoff.

Patentansprüche

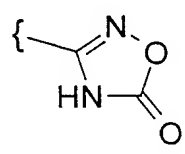
1. Verbindungen der Formel I



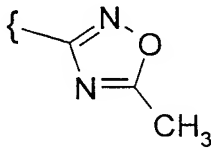
I

worin

R	H, unverzweigtes oder verzweigtes Alkyl mit 1-6 C-Atomen oder Cycloalkyl mit 3-6 C-Atomen,
R^1	Ar,
R^2	Ar',
R^3	H, R, R^4, Hal, CN, COOH, COOA oder $CONH_2$,
Ar, Ar'	jeweils unabhängig voneinander unsubstituiertes oder ein-, zwei- oder dreifach durch R, OH, Hal, CN, NO_2, CF_3, NH_2, NHR, NR_2, Pyrrolidin-1-yl, Piperidin-1-yl, Benzyloxy, SO_2NH_2, SO_2NHR, SO_2NR_2, -CONHR, $-CONR_2$, $-(CH_2)_n-NH_2$, $-(CH_2)_n-NHR$, $-(CH_2)_n-NR_2$, $-O-(CH_2)_n-NH_2$, $-O-(CH_2)_n-NHR$, $-O-(CH_2)_n-NR_2$, R^4 oder zusammen durch $-O-(CH_2)_m-O-$ substituiertes Phenyl, Naphthyl oder Biphenyl,
R^4	unsubstituiertes oder einfach durch -COR, -COOR, -OH oder durch eine konventionelle Aminoschutzgruppe substituiertes $-C(=NH)-NH_2$ oder $-NH-C(=NH)-NH_2$, $-C(=O)-N=C(NH_2)_2$,



oder



,

A	Alkyl mit 1-4 C-Atomen,
Hal	F, Cl, Br oder I,

m 1 oder 2,
n 0, 1, 2 oder 3,
p 0 oder 1 bedeutet,
sowie deren Salze.

2. Verbindungen gemäß Anspruch 1

a) 5-(3-Amidino-benzyl)-3-(7-amidino-naphth-2-ylmethyl)-2-isopropyl-3,5-dihydro-imidazo[4,5-*c*]-pyridin-4-on;

b) 3,5-Bis-(7-amidino-naphth-2-ylmethyl)-2-isopropyl-3,5-dihydro-imidazo[4,5-*c*]-pyridin-4-on;

sowie deren Salze.

3. Verfahren zur Herstellung von Verbindungen der Formel I nach Anspruch 1 sowie ihrer Salze, dadurch gekennzeichnet, daß man

a) sie aus einem ihrer funktionellen Derivate durch Behandeln mit einem solvolysierenden oder hydrogenolysierenden Mittel in Freiheit setzt, indem man

i) eine Amidinogruppe aus ihrem Oxadiazolderivat oder Oxazolidinonderivat durch Hydrogenolyse oder Solvolyse freisetzt,

ii) eine konventionelle Aminoschutzgruppe durch Behandeln mit einem solvolysierenden oder hydrogenolysierenden Mittel durch Wasserstoff ersetzt oder
eine durch eine konventionelle Schutzgruppe geschützte Aminogruppe in Freiheit setzt,

oder

b) in einer Verbindung der Formel I einen oder mehrere Rest(e) R, R^1, R^2 und/oder R^3 in einen oder mehrere Rest(e) R, R^1, R^2 und/oder R^3 umwandelt,

indem man beispielsweise

i) eine Estergruppe zu einer Carboxygruppe hydrolysiert,

ii) eine Nitrogruppe reduziert,

iii) eine Aminogruppe acyliert,

iv) eine Cyangruppe in eine Amidinogruppe

und/oder

c) eine Base oder Säure der Formel I in eines ihrer Salze umwandelt.

4. Verfahren zur Herstellung pharmazeutischer Zubereitungen, dadurch gekennzeichnet, daß man eine Verbindung der Formel I nach Anspruch 1 und/oder eines ihrer physiologischen unbedenklichen Salze zusammen mit mindestens einem festen, flüssigen oder halbflüssigen Träger- oder Hilfsstoff in eine geeignete Dosierungsform bringt.

5. Pharmazeutische Zubereitung, gekennzeichnet durch einen Gehalt an mindestens einer Verbindung der Formel I nach Anspruch 1 und/oder einem ihrer physiologisch unbedenklichen Salze.

6. Verbindungen der Formel I nach Anspruch 1 und ihre physiologisch unbedenklichen Salze oder Solvate als Arzneimittelwirkstoffe.

7. Verbindungen der Formel I nach Anspruch 1 und ihre physiologisch unbedenklichen Salze zur Bekämpfung von Thrombosen, myocar-

dialem Infarkt, Arteriosklerose, Entzündungen, Apoplexie, Angina pectoris, Restenose nach Angioplastie und Claudicatio intermittens.

8. Arzneimittel der Formel I nach Anspruch 1 und ihre physiologisch unbedenklichen Salze als Inhibitoren des Koagulationsfaktors Xa.

9. Verwendung von Verbindungen der Formel I nach Anspruch 1 und/oder ihre physiologisch unbedenklichen Salze zur Herstellung eines Arzneimittels.

10. Verwendung von Verbindungen der Formel I nach Anspruch 1 und/oder ihrer physiologisch unbedenklichen Salze zur Herstellung eines Arzneimittels zur Bekämpfung von Thrombosen, myocardialem Infarkt, Arteriosklerose, Entzündungen, Apoplexie, Angina pectoris, Restenose nach Angioplastie und Claudicatio intermittens.

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/EP 99/06655

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07D471/04 A61K31/435 //(C07D471/04,235:00,221:00)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07D A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 January 2000	23/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Steendijk, M

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/06655

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
IPK 7 C07D471/04 A61K31/435 //(C07D471/04,235:00,221:00)

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)
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C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie°	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
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[X] Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

[X] Siehe Anhang Patentfamilie

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28. Januar 2000	23/02/2000

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Europäisches Patentamt, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Steendijk, M

3

Formblatt PCT/ISA/210 (Blatt 2) (Juli 1992)

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Internales Aktenzeichen
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C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

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(51)4 C 07 D 471/04; A 61 K 31/395

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DESCRIPTION OF AN INVENTION
FOR AN AUTHORSHIP CERTIFICATE

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(22) March 30, 1981

(46) December 23, 1986, Bulletin No. 47

(71) Institute of Physicoorganic Chemistry and Coal Chemistry of the Ukrainian SSR Academy of Sciences, and the Zaporozh'ye State Medical Institute

(72) Yu. M. Yutilov, A. G. Ignatenko, L. Ye. Mikhailova, and V. V. Kirichenko

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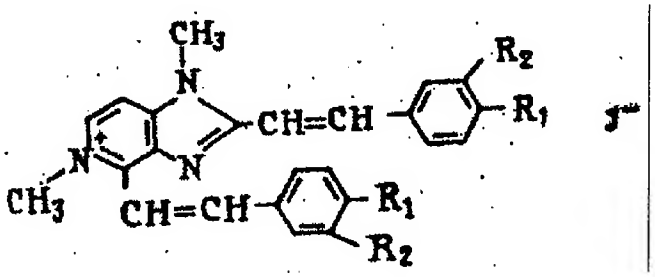
(54) 2,4-DISTYRYL DERIVATIVES OF IMIDAZO[4,5-*c*]PYRIDINIUM EXHIBITING BACTERIOSTATIC AND FUNGISTATIC ACTIVITY

(57) 2,4-Distyryl derivatives of imidazo[4,5-*c*]pyridinium having the general formula



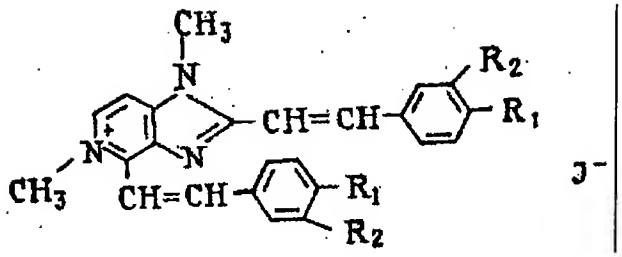
where a) $R_1 = N(CH_3)_2$ and $R_2 = H$, and b) $R_1 = R_2 = OCH_3$,
which exhibit bacteriostatic and fungistatic activity.

The invention relates to new chemical compounds of the imidazopyridine series, specifically to 2,4-distyryl derivatives of imidazo[4,5-*c*]-pyridinium, which exhibit bacteriostatic and fungistatic activity and can be used in the chemical-pharmaceutical industry.

5-Dodecyl-1-methylimidazo[4,5-*c*]-pyridinium bromide, which exhibits antimicrobial and fungistatic activity, has been described in the patent literature. However, it has high toxicity (LD_{50} = 13 mg/kg).

The object of the invention is to expand the toolkit for acting on the living organism.

The stated object is attained by the described 2,4-distyryl derivatives of imidazo[4,5-c]-pyridinium having the general formula:



where a) $R_1 = N(CH_3)_2$ and $R_2 = H$, and b) $R_1 = R_2 = OCH_3$,
which are obtained by reacting 1,2,4,5-tetramethylimidazo[4,5-*c*]pyridinium iodide with an excess of the corresponding aromatic aldehyde in the presence of piperidine as a catalyst.

Example 1. 2,4-Di-(*n*-N′,N′-dimethylaminostyryl)-1,5-dimethylimidazo[4,5-*c*]pyridinium iodide (1a).

A quantity of 0.1 g (3.3×10^{-4} mol) of 1,2,4,5-tetramethylimidazo[4,5-*c*]pyridinium iodide and 0.2 g (13.2×10^{-4} mol) of *n*-N,N-dimethylaminobenzaldehyde is dissolved, while being heated in 5 mL of *n*-butanol, 0.24 mL (2.4×10^{-4} mol) of piperidine is added, and [the resulting mixture] is boiled on an oil bath at a temperature of 135–145°C for 3.5 hr. After cooling, the cherry-colored precipitate is filtered off and washed with ether; the yield is 0.072 g (38.6%), and m.p. is 250°C with decomposition (*n*-butanol).

EPR spectrum, δ, ppm (CF_3COOH): 3.02 [*c*, $-(CH_3)_2$]; 3.92 [*c*, 1(5)–CH_3]; 4.12 [*c*, 5(1)–CH_3]; 7.30–7.55 (*m*, $-C_6H_4-$ and –CH=CH–); 7.72 [*d*, 7(6)–H, I = 6.5 Hz]; 8.43 [*d*, 6(7)–H, I = 6.5 Hz].

Found: C 59.7%; H 6.0 %; N 22.3%.

$C_{28}H_{32}N_5I$.

Calculated: C 59.5%; H 5.7%; N 22.4%.

Example 2. 2,4-Di-(3,4-dimethoxystyryl)-1,5-dimethylimidazo-[4,5-*c*]-pyridinium iodide (1b).

[This compound] is obtained, by analogy with Example 1, by proceeding from 0.1 g (3.3×10^{-4} mol) of 1,2,4,5-tetramethylimidazo[4,5-*c*]pyridinium iodide and 0.25 g (1.5×10^{-4} mol) of 3,4-dimethoxybenzaldehyde; yield is 0.15 g (75.8%) of a substance of light-brown color, and m.p. is 175–176°C (*n*-butanol).

EPR spectrum (CF_3COOH, δ, ppm): 3.60 (*c*, 2,5-OCH_3); 3.89 [*c*, 1(5)-CH_3]; 4.09 [*c*, 5(1)–CH_3]; 6.6–7.23 (*m*, $-C_6H_3-$ and –CH=CH–); 7.69 [*d*, 7(6)–H, I = 6.5 Hz]; 8.33 (*d*, 6(7)–H, I = 6.5 Hz).

Found: C 55.8%; H 5.2%; N 20.9%.

$C_{28}H_{30}N_3IO_4$.

Calculated: C 56.1%; H 5.0%; N 21.2%.

The bacteriostatic activity of the compounds was studied by the method of doubling dilutions on a liquid medium. Hottinger broth (*p*H 7.2–7.4) was used to culture the bacteria. The microbial load for the bacteria was 5×10^5 cells of an 18-hr agar culture in 1 mL of medium. The highest of the tested concentrations was 200 μg/mL.

Sabouraud's medium (*p*H 6.0–6.8) was used to grow the fungi. The load was 500,000 reproductive bodies per milliliter. The highest of the tested concentrations was 200 μg/mL. The antimicrobial activity of the compounds [was determined] from the minimum bacteriostatic or mycostatic concentration of chemical compounds, expressed in μg/mL.

The test results for activity and toxicity are presented in the table.

Thus, 2,4-distyryl derivatives of imidazo[4,5-*c*]-pyridinium having general formula 1 possess a broader spectrum of bacteriostatic and fungistatic activity than does 5-dodecyl-1-methylimidazo[4,5-*c*]pyridinium bromide, and also are less toxic compounds.

Test Results for Antimicrobial and Fungistatic Activity
(the minimum bacteriostatic and mycostatic concentrations are specified in μg/mL)

Strain of microorganisms and fungi	**2,4-Distyryl derivatives of imidazo[4,5-c]-pyridinium**	
	1a	**1b**
Staphylococcus aureus 209 P	100	>200
Escherichia coli 675	200	>200
Shigella Flexneri	50	200
Bacillus anthracoides 1312	6.25	200
Microsporum lanosum 257	50	200
Trichophyton mentag. IMI 124768	50	200
Aspergillus niger BKMF-1119	200	>200
Toxicity, LD_{50} (mg/kg)	44.7 ± 6.05	48.7 ± 2.67

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(71) Applicant *(for all designated countries except US)*: RHONE POULENC CHIMIE [FR]; BRANLARD PAUL [FR]; PRIOU CHRISTIAN [FR]; VAULTIER MICHEL [FR]

(72) Inventor; and

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(74) Attorney: MONCHENY, Michel; Cabinet Lavoix, 2, place d'Estienne d'Orves, F-75441 Paris Cedex 09 (FR).

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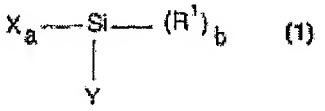
(54) Title: Silanes and Polyorganosiloxanes with boronate function(s)



(1)

(2)

(57) Abstract

The invention concerns silanes of formula (1) wherein Y is a boronate group of formula (2), wherein: c and d are integers ranging from 0 to 18; c + d = 0 to 18; e is selected between 0 and 1; Z is a divalent heterocarbon group comprising one or several heteroatoms such as O, S, and/or N. The invention also concerns the corresponding polyorganosiloxanes, the methods for preparing them and compositions containing them.

Silanes and Polyorganosiloxanes with boronate function(s)

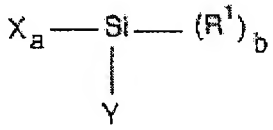
The present invention concerns novel functionalized silanes and polyorganosiloxanes, as well as compositions comprising a polyorganosiloxane of this type as a base polymer, capable of crosslinking into an elastomer through exposure to ambient air humidity without the aid of a crosslinking catalyst.

It also concerns the preparation processes of these silanes and polyorganosiloxanes.

Single-component silicone compositions (that is, in the form of a single package) capable of hardening or crosslinking via polycondensation reaction through exposure to ambient air humidity at room temperature are well known to those skilled in the art and are described in numerous patent documents. In this context one generally utilizes a tin base catalyst capable of generating degradation reactions of the elastomer formed during age-hardening of the latter.

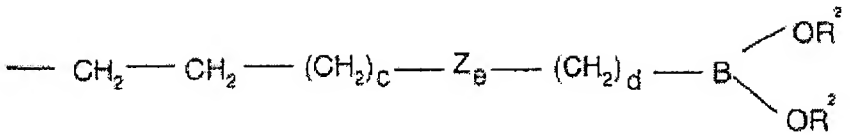
The main objective of the invention is to propose a polyorganosiloxane-based silicone composition for thin- and thick-film coating that is storage-stable in the absence of humidity and hardenable at room-temperature without the aid of a crosslinking catalyst.

A first object of the invention concerns the silicones that are used to prepare the polyorganosiloxanes according to the invention and which correspond to the formula (1):



wherein:

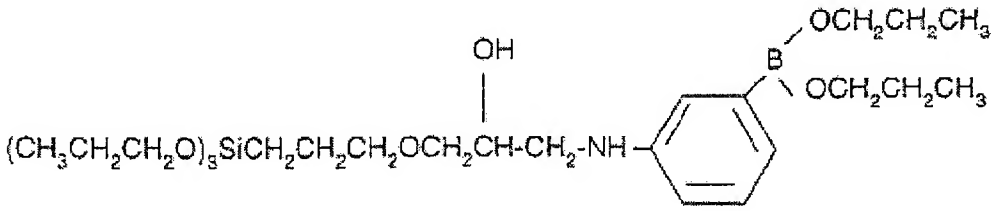
- R^1 is selected from a monovalent hydrocarbonate group, possibly substituted by halogen atoms,
- X is a hydrolysable group,
- a is selected between 1, 2 and 3,
- b is selected between 0, 1 and 2,
- a + b = 3
- Y is a boronate group of formula (2)



wherein:

- c and d are integers ranging from 0 to 18
- c + d = 0 to 18
- e is selected between 0 and 1,
- Z is a divalent heterocarbon group comprising one or several heteroatoms such as O, S and/or N; well suited are: -O-, -CO-, -COO-, phenylene, -NR'- with R' = H or straight-chain or branched-chain C1-C4, -S- alkyl
- R^2 groupings, which can be identical or different, are selected from hydrogen atoms, straight or branched-chain C1-C20 alkyl radicals, preferably C1-C6, C5-C8 cycloalkyls, C6-C12 aryls, or with boron and oxygen atoms can form a heterocycle consisting of 5 to 8 elements (heterocycle atoms), preferably 5 to 6; the carbons may possibly be substituted,

being excluded the silane of formula



This particular silane, excluded by disclaimer, was disclosed by M. Glad et al., J. Chromato., 1985, 347: 11-23. It is used in the field of chromatography to produce a polysiloxane coating on porous silica to enable molecular imprinting or enzyme fixation.

Preferably, in formula (2), one will select:

either: - c + d = 0 or 1
- e = 0
- R^2 = straight- or branched-chain C1-C4 and/or H alkyl groupings,

or: - c = 0 or 1
- d = 0
- e = 1
- Z is a phenyl group
- R^2 = straight- or branched-chain C1-C4 and/or H alkyl groupings.

Preferably, in formula (1), the radicals R^1, identical or different, are C1-C10 hydrocarbon radicals, substituted or unsubstituted by halogen atoms.

These radicals notably comprise:

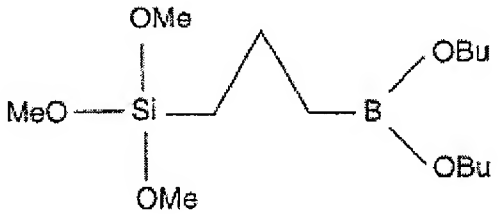
- C1-C10 alkyl and halogen alkyl radicals, such as the radicals: methyl, ethyl, propyl, isopropyl, butyl, pentyl, hexyl, ethyl-2 hexyl, octyl, decyl, trifluoro-3,3,3 propyl, trifluoro-4,4,4 butyl, pentafluoro-4,4,4, 3,3 butyl,
- C3-C10 cycloalkyls and halogenocycloalkyls, preferably C5-C8, such as the radicals: cyclopentyl, cyclohexyl, methylcyclohexyl, propylcyclohexyl, difluoro-2,3 cyclobutyl, difluoro-3,4 methyl-5 cycloheptyl,
- C2-C4 alkenyl radicals, such as the radicals: vinyl, allyl, butene-2-yl,
- mononuclear C6-C10 aryl and halogen aryl radicals, such as the radicals: phenyl, tolyl, xylyl, chlorophenyl, dichlorophenyl, trichlorophenyl.

The preferred radicals are methyl, phenyl, vinyl and trifluoro-3,3,3.

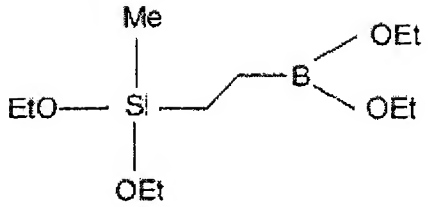
The hydrolyzable X radicals, identical or different, are more specifically selected from a halogen atom (preferably chlorine) and among N-substituted amino radicals: N-substituted amido, N,N di-substituted aminoxy, cetiminoxy, aldimonoxy, alkoxy, alkoxy alkylene-oxy, enoxy, acyloxy. For more details concerning the X radicals that can be used, those skilled in the art may refer to EP-B1-430 826.

Methoxy, ethoxy and acetoxy radicals are particularly suitable.

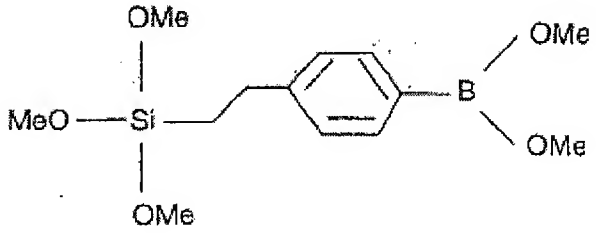
As preferred silanes, one may cite the following:



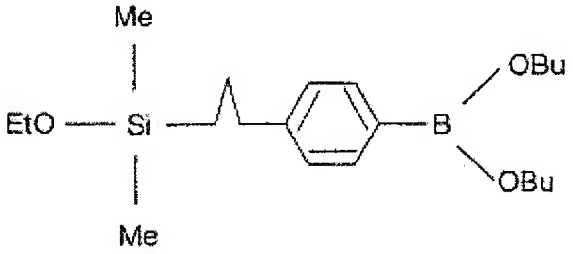
(A)



(B)



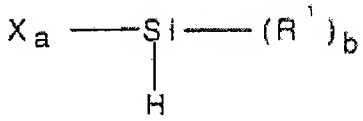
(C)



(D)

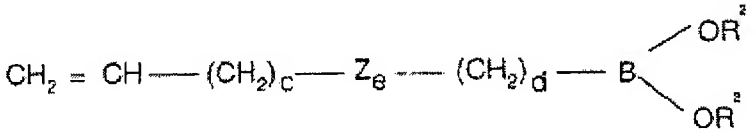
The silanes can be prepared via hydrosilylation reaction between:

- a silane of formula (3)



wherein: X, a, R^1 and b have the same meanings as above, and

- an unsaturated alkyl dialkoxy borane of formula (4)



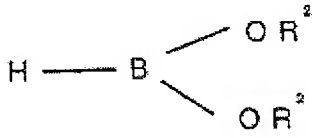
wherein:

- c and d are integers ranging from 0 to 18
- c + d is at the most equal to 18
- R^2, Z and e have the same meanings as above.

This reaction is carried out in the presence of a conventional hydrosilylation catalyst such as rhodium, platinum, in the presence possibly of an inert solvent such as toluene or cyclohexane, at a temperature ranging between room temperature and 120°C.

Preferably, the unsaturated alkyl dialkoxy borane is such that c = 0 or 1, e = d = 0.

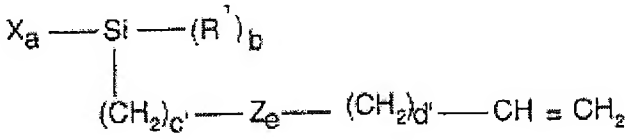
Certain silanes can also be prepared by hydroboration reaction by causing to react together:



(5),

wherein R_2 is as above, and:

(6)



wherein:

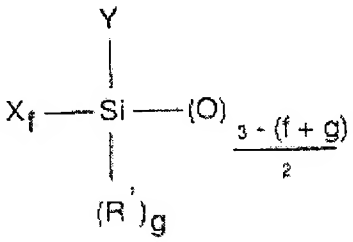
- c = 2 to 18
- d' = 0 to 16
- c' + d' = 2 to 18
- R^1, b, X, a, Z, e have the same meanings as above,
- with the possibility, when e = 0, that c' = 0 and d' = 0 or 1 (c' + d' = 0 or 1).

This reaction is carried out with or without catalyst, possibly in the presence of a solvent like cyclohexane, at a temperature ranging from -50°C to +100°C.

As preferred examples, functionalized silanes according to the invention can be prepared through hydrosilylation of a vinyl dialkoxy borane (c = 0, formula (4)) or an allyl dialkoxy borane (c = 1, formula (4)), or through hydroboration of a vinyl silane (c' = d' = e = 0, formula (6)), or an allyl silane (c' = 1, e = d' = 0, formula (6)). One may further specify that these alkoxy groupings are preferably methoxy, ethoxy, propyloxy, butyloxy, pentyloxy or hexyloxy groupings. Allyl dibutoxy borane, also known as butyl allyl boronate, will be mentioned as a suitable example, where in formula (4) c = 1, d = e = 0, R^2 = butyl.

The functionalized silanes of formula (1) can be used as such, notably as adherence promoters in elastomeric compositions or as a crosslinking agent in crosslinking polycondensation reactions for silicone compositions (preferably in that case silanes (1) where a = 2 or 3).

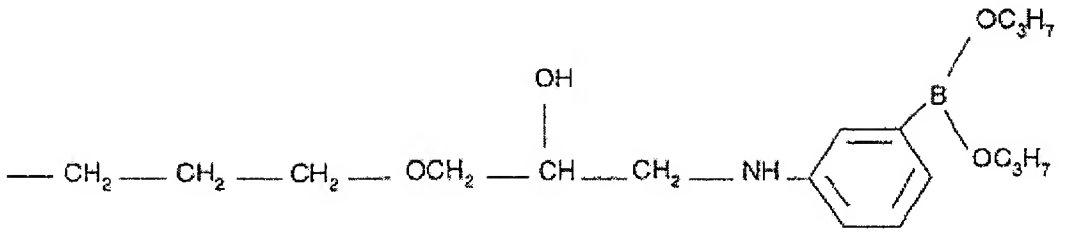
The second object of the invention also concerns polyfunctional polyorganosiloxanes comprising per molecule at least one unit corresponding to the general formula (7):



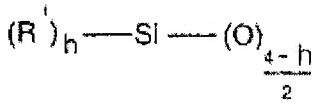
wherein:

- R^1, X and Y have the same meaning as in formula (1),
- f is selected from among 0, 1 or 2,
- g is selected from among 0, 1 or 2,
- f + g is at most equal to 2,

being excluded the polyorganosiloxane in which f + g = 0 and Y is such that:



The polyfunctional polyorganosiloxane can have, on the one hand, at least one unit (7) taken without its disclaimer and, on the other, other siloxyl units corresponding to the formula (8):



wherein:

- R^1 has the same meaning as formula (1)
- h is selected from among 0, 1, 2, and 3.

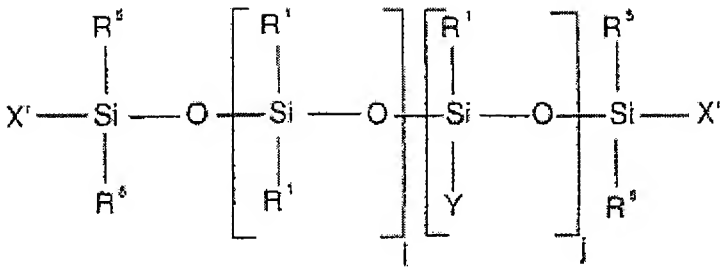
Preferably, R^1 is selected from the radicals: methyl, phenyl and vinyl, at least 80% by number of the R1 radicals being methyl.

The polyorganosiloxanes according to the invention can therefore have a straight-chain, cyclic or branched-chain structure.

Preferred are straight-chain or cyclic polyorganosiloxanes having per molecule at least one unit of formula (7) where f + g is different from zero, and possibly at least one unit of formula (8) where h is equal to 2 or 3.

Such straight-chain or cyclic polymers can possibly include T units of formula (7) where f + g = 0 and/or T units of formula (8) where h = 1 and/or possibly Q units of formula (8) where h = 0 in a proportion of at least 2% (these % expressing the number of T and/or Q units per 100 atoms of silicon).

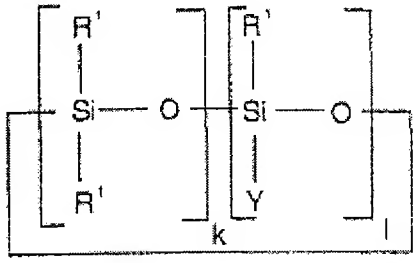
The present invention notably concerns the polyfunctional polyorganosiloxanes of formula (9):



wherein:

- R^1, X and Y have the same meaning as in formula (1) [handwritten: without its disclaimer],
- X' is selected from among the radicals Y, R^1, hydroxyl and hydrogen atom,
- the radicals R^5, identical or different, are selected from the radicals R^1 and X
- i is an integer between 0 and 1000,
- j is an integer between 0 and 50,
- if j = 0, at least 1 of the radicals X^1 is Y.

The invention also concerns the polyorganosiloxanes of formula (10).



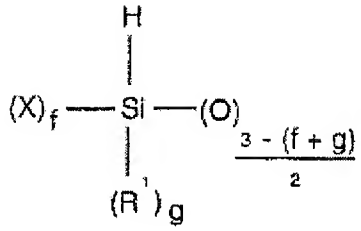
wherein:

- R^1 and Y have the same meaning as in formula (1) [handwritten: without its disclaimer],
- k is an integer between 0 and 9, inclusive,
- l is an integer between 1 and 9, inclusive,
- k + l is between 3 and 10, inclusive.

These polyfunctional polyorganosiloxanes can be prepared according to different processes.

A first process consists in bringing about a hydrosilylation reaction between:

- a polyorganosiloxane having per molecule at least one unit of formula (11):

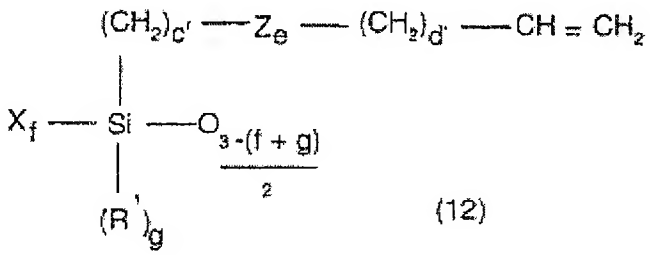


wherein X, f, R^1, g have the same meanings as in formula (7), without its disclaimer, and

- an unsaturated alky dialkoxy borane of formula (4), above.

Preferably, the compound of formula (4) is such that c = 0 or 1 and e = d = 0.

Certain polyorganosiloxanes can be prepared via hydroboration reaction between the compound of formula (5), and:



(12)

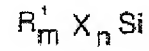
wherein c' = 2 to 18

d' = 0 to 16

c' + d' = 2 to 18

formulas (12) and (12') wherein X, f, R^1, g and Z have the same meanings as above, with the possibility, when e = 0, that c' = 0 and d' = 0 or 1 (c' + d' = 0 or 1).

Yet another process consists in producing the polyorganosiloxane via hydrolysis and/or redistribution reactions from a functionalized silane of formula (1) according to the invention with a cyclic or branched-chain polysiloxane comprising units of formula (8) wherein h = 2 or 3, or with a hydrolyzable silane corresponding to formula (13):



wherein:

- R^1 has the same meaning as formula (1),
- m = 2 or 3,

- $m + n = 4$,
- X as described in formula (1).

In the case where polycondensation is stopped by neutralization, one obtains a reaction mixture comprising cyclic polymers of formula (10) and/or branched polymers of formula (9) blocked at each of their ends by a hydroxyl group or by the unit:

$R^1_2YSiO_{0,5}$ if at the start one additionally utilizes the silane R^1_2YSiCl or the corresponding disiloxane.

One can also stop the polycondensation by adding, at the end of reaction, an organosilicon compound susceptible to reacting with the terminal hydroxyls of the formula (9) polymer formed, whereby this organosilicon compound corresponds to the formulas:

R^1_3SiCl, $R^1_3SiNHSiR^1_3$ and $R^1_3SiOSiR^1_3$.

Hydrolysis duration can vary between a few seconds and several hours.

After hydrolysis, the aqueous phase is separated from the siloxane phase by any suitable physical means, usually by decantation and/or extraction via an organic solvent like isopropyl ether or toluene.

In the presence of humidity, the polyfunctional polyorganosiloxanes of the invention crosslink to form very UV- and temperature-stable boroxines. They can also be placed in aqueous solution in the form of stable boronic acid, crosslinkable in boroxine via elimination of water.

These polyorganosiloxanes can be utilized pure to form, for example, coatings after crosslinking, in the absence of a catalyst, in the presence of humidity or by elimination of water, as required.

They are particularly usable as base diorganopolysiloxane polymers, within a silicone compound crosslinkable into an elastomer, in the absence of a catalyst, through exposure to atmospheric humidity or through elimination of water, depending on the case.

These silicone compounds can be single-packaged and are storage-stable.

In a third object, the invention thus concerns an organopolysiloxane compound, storage-stable in the absence of humidity and capable of crosslinking through exposure to humidity in the absence of a crosslinking catalyst, comprising:

(A) – 100 parts by weight of at least one polymer of formula (7) without its disclaimer, possibly with units (8), or of formula (9) or (10), according to the invention

(B) – 0 to 250 parts by weight of a mineral filler.

It therefore also concerns an aqueous organopolysiloxane compound, storage-stable in the absence of humidity and capable of crosslinking through elimination of water in the absence of a crosslinking catalyst, comprising:

(A) – 100 parts by weight of at least one polymer, a polymer of formula (7) without its disclaimer, possibly with units (8), or of formula (9) or (10), according to the invention

(B) – 0 to 250 parts by weight of a mineral filler.

(C) – 0.5 to 50, preferably 3 to 20, parts by weight of water.

(D)– possibly a non-ionic, anionic, cationic or amphoteric tensioactive agent.

Mineral fillers (B) are used at the rate of 0 to 250 parts, preferably 20 to 200 parts, per 100 parts of polymer (A).

These fillers can be in the form of very finely divided products whose mean particle diameter is less than 0.1 micrometer. These fillers include combustion silica and precipitated silica; their specific BET surface is generally greater than 40 m^2/g.

These fillers can also be in the form of more coarsely divided products whose mean particle diameter is greater than 0.1 micrometer. As examples of such fillers, one may cite milled quartz, diatomaceous silica, calcium carbonate, calcined clay, titanium oxide of the rutyl type, ferrous, zinc, chromium, zirconium, magnesium oxides, various forms of aluminum (hydrated or unhydrated), boron nitride, barium metaborate, glass microbeads; their specific surface is generally less than 30 m^2/g.

These fillers (B) can be surface-modified through treatment with various organosilicon compounds customarily used for this purpose. Accordingly, these organosilicon compounds can be organochlorosilanes, diorganocyclopolysiloxanes, hexorganodisiloxanes, hexorganodisilazanes or diorganocyclopolysiloxanes (French patents FR-A-1 126 884, FR-A-1 136 885, FR-A-1 236 505; English patent GB-A-1 024 234). In the majority of cases, the treated fillers are comprised from 3 to 30% by weight of organosilicon compounds.

The fillers (B) can consist of a mixture of several types of fillers of varying granulometry.

The tensioactive agents (D) possibly utilized can be nonionic tensioactives with an HLB greater than 10, preferably on the order of 10 to 20, anionic, cationic, zwitterionic or amphoteric agents with an HLB greater than 10.

The nonionic tensioactive agents can be selected from alkoxylated fatty acids, polyalkoxylated alkyphenols, polyalkoxylated fatty alcohols, polyalkoxylated or polyglycerolated fatty amides, polyglycerolated alcohols and alphadiols, ethylene oxide-propylene oxide block polymers, as well as alkyl glucosides, alkyl polyglucosides, sucroethers, sucroesters,

sucroglycerides, sorbitan esters, and the ethoxylated compounds of these sugar derivates having an HLB of at least 10.

The anionic tensioactive agents can be selected from alkyl benzene sulfonates, alkyl sulphates, alkyl ether sulfates, alkyl aryl ether sulfates, dialkyl sulfosuccinates, alkyl phosphates, ether phosphates, alkaline metals having an HLB of at least 10.

Among the cationic tensioactive agents one may cite aliphatic or aromatic fatty amines, aliphatic fatty amines, quaternary ammonium derivates, having an HLB of at least 10.

Among the zwitterionic or amphoteric tensioactive agents one may cite betaines and their derivates, sultaines and their derivates, lecithins, imidazoline derivates, glycinates and their derivates, amino propionates, fatty amine oxides having an HLB of at least 10.

The polyorganosiloxanes with boronate functions according to the invention can be utilized in the pure state or in the form of compounds of the type of those cited above, for example in the field of textile conditioning and in the coating of metals, natural stones or various cement-based construction equipment in order to confer nonadherent and/or hydrophobic surface properties to same.

The invention therefore likewise concerns this use as well as the method of treating these different materials or substrates, in which these substrates are coated with the polyorganosiloxanes or compounds according to the invention, in order to confer to these substrates, after crosslinking, adherent and/or hydrophobic properties.

The invention will now be described in greater detail with the aid of non-limiting exemplified embodiments.

Silanes and vinyl silanes are industrial and commercial products. The methods of boron hydride preparation are known. One may cite Jeffers P.M. et al., Inorg. Chem., 1981, 20:1698 for the preparation of $HB(OMe)_2$. The synthesis of allyl dibutoxy borane is described below.

EXAMPLES

Example 1: Synthesis of allyl dibutoxy borane:

Into a 1 liter three-necked flask equipped with a mechanical agitator, an ascendant refrigerant, a 100 ml isobaric funnel, an N_2 valve (all equipment flame-dried under N_2), introduce 0.5 moles of Mg, then freshly distilled BF_3 etherate and 350 ml of anhydrous ether distilled over Na.

Under strong agitation at room temperature, add dropwise a 28.62 g solution of allyl chloride in 50 ml of anhydrous ether. The reaction is triggered through direct introduction of 4 cm^3 (over the 28.60 g) of allyl chloride into the reactor.

The ether rapidly refluxes and, once returned to a reasonable level, is maintained by addition of an allyl chloride solution, under vigorous agitation.

After 1 hour and 30 minutes of addition, the solution is left at room temperature for 2 hours, under vigorous agitation.

Following decantation, the supernatant is transferred under N_2 into a degassed and dry flask for 1 hour.

Add 3 x 200 ml of anhydrous ether over the precipitate under agitation in order to extract the maximum product amount from it. The supernatants formed, the ether is distilled at atmospheric pressure. After transfer under N_2 of the product remaining in the 100 ml flask, the triallyl-borane is distilled at 60°C for 20 minutes.

Yield: 67%.

Into a 100 mg double-neck flask, equipped with magnetic agitation and a refrigerant + valve, all equipment flame-dried under argon, introduce 9 g of freshly distilled triallyl-borane.

Add dropwise an excess of 1-butanol freshly distilled under N_2. The reaction is exothermic. If required, provide water bath cooling at 30-35°C.

Leave one night at room temperature. Then distill the excess of 1-butanol under vacuum at 40 mm Hg (5320 Pa), after that the anticipated product under vacuum at 0.2 to 0.4 mm Hg (26.6 to 53.2 Pa) at 58-62°C; 12.2 g of a colorless liquid is obtained. A slight residue remains. The residue and liquid are collected and redistilled through a 13 cm Vigreux column. The anticipated product (allyl dibutoxy borane) is recovered at 50°C - 52°C under vacuum at 0.1 mmHg (13.3 Pa).

Note: It is possible to not purify the triallyl-borane intermediate and to immediately add butanol.

Example 2: Hydrosilylation of an Si-H oil by the allyl dibutoxy borane obtained in Example 1:

Reagents:

- 7.18 g of polyhydrogenosiloxane oil ($MD_9D'_4M$ [where M = $(CH_3)_3SiO_{1/2}$, D = $(CH_3)_2SiO_{2/2}$ and D' = $H(CH_3)SiO_{2/2}$] having 384 millimoles of SiH units per 100 g of oil and viscosity of 12 mPa·s at 25°C (Brookfield viscometer)
- 6.55 g of allyl dibutoxy borane (0.033 mmoles)
- 10 ppm of Karstedt-type platinum catalyst: solution in the divinyl tetramethyl disiloxane of a platinum compound at approximately 11% by weight of zero-valent platinum liganded by

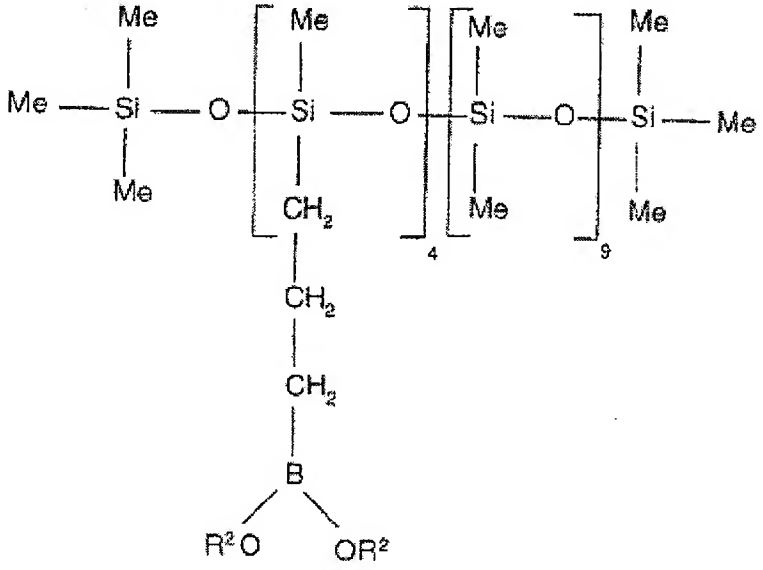
divinyl tetramethyl disiloxane: the quantities of this catalyst are expressed in ppm of Pt metal supplied by the solution in the reaction mixture.

Procedure:

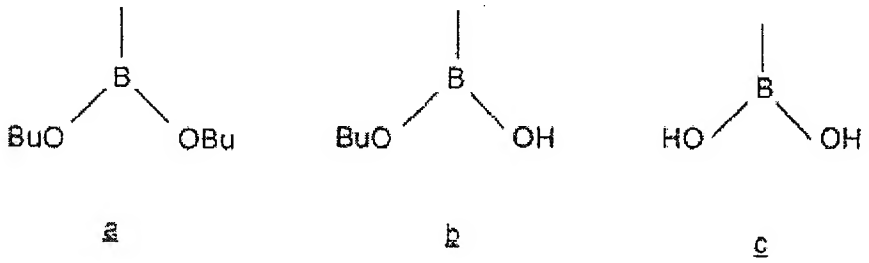
Pour the oil into an isobaric funnel. Introduce the allyl dibutoxy borane and the catalyst into the flask (100 ml three-necked with mechanical agitator). Slowly pour the oil over the alkene. Then heat the reaction medium in the range of 70-75°C for 2 hours.

The IR spectrometer allows reaction progress to be followed by monitoring the disappearance of the SiH band (in the 2100 cm^{-1} region). At the end of reaction, eliminate the reagents that did not react to heating under vacuum (13.3 10^2 Pa.) in the 110-120°C range.

The polyorganosiloxane obtained corresponds to the following formula:



wherein the boronate functions are constituted essentially by a mixture of functions:



a b c

where the a functions are amply in the majority in number.

Example 3: Hydrosilylation of the triethoxysilane by the allyl dibutoxy borane obtained in Example 1:

Product weights:

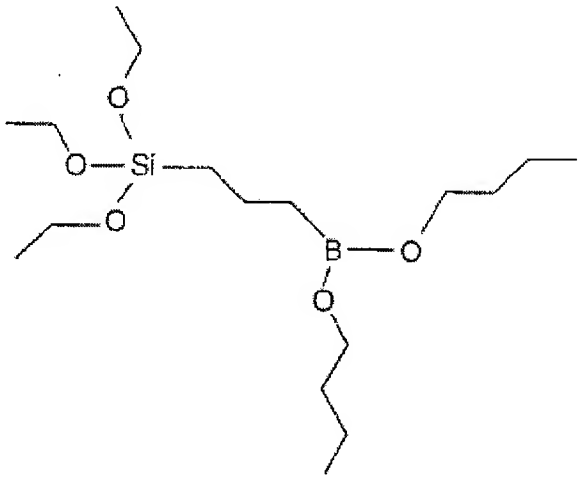
Allyl dibutoxy borane	22.30 g	0.094 mol	1.1 eq.
Triethoxysilane	14.20 g	0.086 mol	1 eq.
Catalyst	0.0009 g		

Procedure:

Into a three-necked flask (dry and under argon), introduce the butyl allyl boronate and Karstedt platinum. Pour the distilled triethoxysilane over this mixture. When finished pouring, heat to 100°C and monitor reaction progress per Si-H dose.

An Si-H dose administered after 24 hours of reaction indicates the reaction has not finished. Re-add catalyst and continue heating. Monitor reaction progress per Si-H dose. At the end of 48 hours, a fourth dose indicates that the transformation rate is 95%.

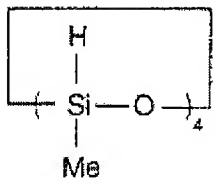
Devolatilize the medium under reduced pressure to eliminate excess butyl allyl boronate. 22 g of product is recovered corresponding to the following formula:



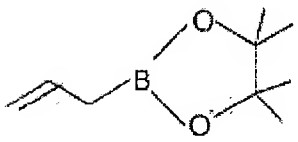
Example 4: Hydrosilylation of allyl pinacol boronate with a cyclic polyorganosiloxane in order to produce 1,3,5,7-tetra-(pinacolboronate)propyl)-1,3,5,7-tetramethyl-cyclotetrasiloxane

Starting compounds:

- cyclic polyorganosiloxane:



- allyl pinacol boronate:



This compound is prepared according to the method described in R.W. Hoffmann et al., Liebigs Ann. Chem. 1986, 1823-1836 or in W.R. Roush et al., J. Am. Chem. Soc. 1986, 108, 3422-3434.

- Karstedt catalyst

Procedure:

Under argon, mix and heat to 60°C allyl pinacol boronate (1.5 ml, 9.1 mmoles) and Karstedt catalyst (1.10^{-4} moles of platinum per mole of allyl pinacol boronate) in toluene (5 ml). After agitation, add the cyclic silane (0.5 ml, 2.1 mmoles). Then heat the solution to 60°C for 4 hours. The solvent is evaporated under reduced pressure and the volatile by-products and excess allyl pinacol boronate extracted through kugelrohr distillation (90% C, 0.2 mm Hg, or 26.6 Pa).

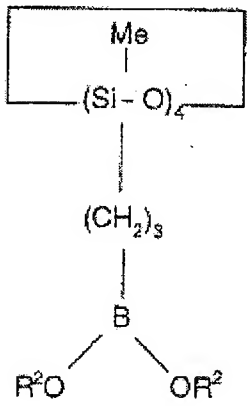
1.31 g of the desired product is obtained.

^{1}H NMR: δ = 0.02 ppm (12H, Si-CH_3), 0.51-0.58 (8H, m, Si-CH_2), 0.74-0.82 (m, 8H, B-CH_2), 1.19 (s, 48H, C-CH_3), 1.41-1.51 (m, 8H, CH_2).

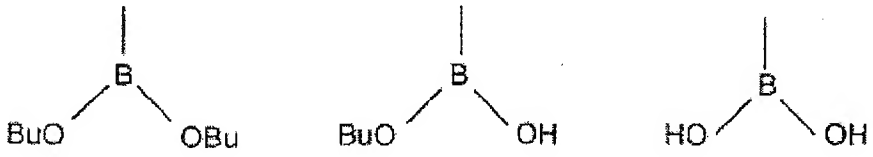
^{13}C NMR: δ = 0.66 ppm (Si-CH_3), 17.53 (Si-CH_2), 19.97 (B-CH_2), 20.24 (CH_2), 24.79 (C-CH_3), 82.73 (C).

Example 5: Hydrosilylation of allyl pinacol borane according to Example 1 with the cyclic polysiloxane of Example 4 in order to obtain 1,3,5,7-tetra-(3-(dibutoxy-boronate)propyl)-1,3,5,7-tetramethyl-cyclotetrasiloxane

Under argon, mix and heat to 70°C allyl dibutoxy borane (1.8 g, 9.1 mmoles) and Karstedt catalyst (1.10^{-4} moles of platinum per mole of allyl dibutoxy borane). After agitation, add the cyclic silane (0.5 ml, 2.1 mmoles). Then heat the solution to 70°C for 4 hours. The solvent is evaporated under reduced pressure and the volatile by-products and excess allyl dibutoxy borane extracted through kugelrohr distillation (100% C/O.2 mm Hg, or 26.6 Pa). 1.6 g of the desired product is obtained having the formula:



wherein the boronate functions are constituted essentially by a mixture of functions:



a b c

where the a functions are amply in the majority in number.

^{1}H NMR: δ = 0.03 ppm (12H, Si-CH_3), 0.48-0.57 (8H, m, Si-CH_2), 0.73-0.81 (m, 8H, B-CH_2), 0.85-0.92 (m, 24H, CH_3-CH_2), 1.24-1.56 (s, 40H, CH_2-CH_2-CH_3, Si-CH_2-CH_2)), 3.72-3.79 (m, 16H, O-CH_2)

^{13}C NMR: δ = 0.65 ppm (Si-CH_3), 13.85 (CH_2-CH_3), 17.71 (Si-CH_2), 19.06, 20.53 (CH_3-CH_2, Si-CH_2-CH_2), 33.81 (O-CH_2-CH_2), 62.92 (O-CH_2).

^{29}Si NMR: δ = -13.80, -20.79, -20.81, -20.87, -20.88, -20.94, -21.00 ppm

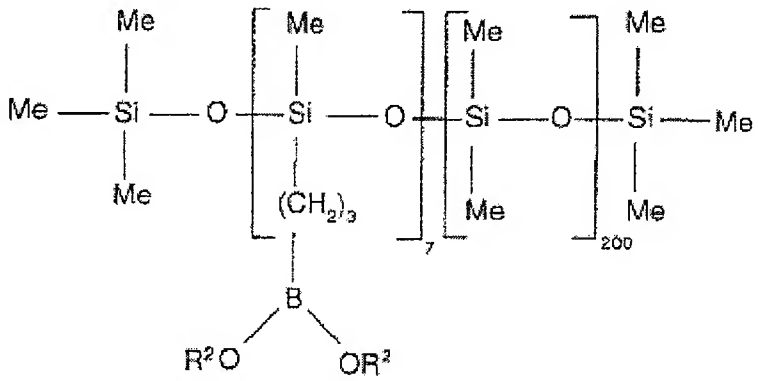
^{11}B NMR: δ = 31.16 ppm

Example 6: Synthesis of allyl diisopropyloxy borane:

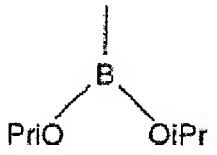
Into a 500 ml reactor under nitrogen, introduce 12 g (0.493 moles) of Mg, 170 ml of anhydrous ether and 20 g (0.141 moles) of BF_3-EtOEt. Then introduce in 1 hour a solution of 32.4 g (0.423 moles) of allyl chloride and 50 ml of dry ether. Start the preparation of magnesium by introducing an iodine crystal. The reaction is exothermic and gives rise to ether reflux. Allow the solution to react 4 hours at room temperature. Bring the reaction mass to 0°C in an ice bath. Then, in 1 hour, add 16.1 g (0.268 moles) of anhydrous isopropanol. Allow the solution to react 35 hours at room temperature. Filter over diatomaceous earth. Evaporate the filtrate and recover the reaction mass which is distilled under vacuum. 13.3 g of $H_2C=CH-CH_2B(OiPr)_2$ is recovered (isolated yield: 55%); boiling temperature: ~ 37°C /760 mmHg (1010.8 10^2 Pa). NMR and IR analyses confirm the structure of this derivate.

Example 7: Hydrosilylation of an Si-H oil by the allyl diisopropyloxy borane obtained in Example 6:

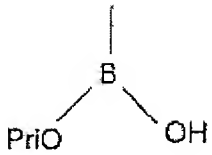
Into a 250 ml reactor under nitrogen, introduce 30 ml of dry toluene and 3.7 g (0.020 moles) of $H_2C=CH-CH_2B(OiPr)_2$. Then add 0.0314 g of the Karstedt catalyst solution (or 104 ppm of Pt metal). Bring the reaction mass to 70°C and in 1 hour pour in $MD_{200}D'_7M$ silicone oil (30 g or 0.0137 moles of SiH units). At the end of 24 hours of reaction at 80°C, the transformation rate of the SiH units is 100%. Thereafter add 2 g of carbon black and leave 1 hour at 60°C. Filter under nitrogen and by devolatilization (100°C, 1-2 mbar, or from 100 to 200 Pa), 35.4 g of a very viscous oil is recovered, the NMR and IR analyses of which confirm the following structure:



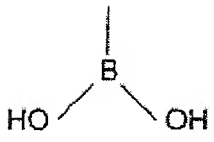
in which structure the boronate functions are constituted essentially by a mixture of functions:



a'



b'

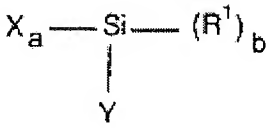


c'

where the a functions are amply in the majority in number.

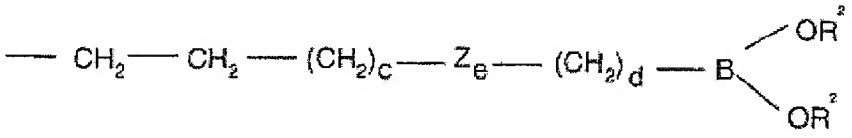
CLAIMS

1. Silane of formula (1)



wherein:

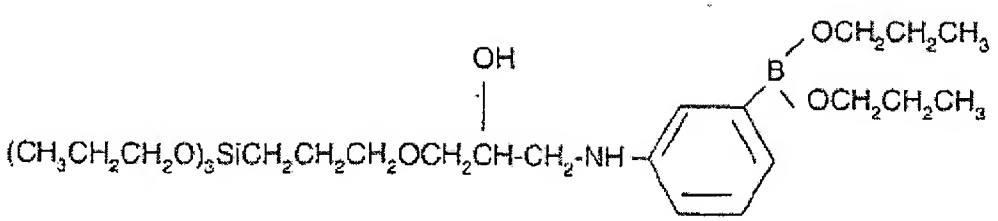
- R^1 is selected from a monovalent hydrocarbonate group, possibly substituted by halogen atoms,
- X is a hydrolyzable group,
- a is selected from among 1, 2 and 3,
- b is selected from among 0, 1 and 2,
- a + b = 3
- Y is a boronate group of formula (2)



wherein:

- c and d are integers ranging from 0 to 18
- c + d = 0 to 18
- e is selected between 0 and 1,
- Z is a divalent heterocarbon group comprising one or several heteroatoms such as O, S and/or N
- R^2 groupings, which can be identical or different, are selected from hydrogen atoms, straight or branched-chain C1-C20 aklyl radicals, preferably C1-C6, C5-C8 cycloalkyls, C6-C12 aryls, or can form with boron and oxygen atoms a heterocycle consisting of 5 to 8 elements (heterocycle atoms), preferably 5 to 6; the carbons may possibly be substituted,

being excluded the silane of formula



2. Silane according to Claim 1, characterized in that, in formula (2),
   - c + d = 0 or 1
   - e = 0
   - R^2 are straight- or branched-chain C1-C4 and/or H alkyl groupings.

3. Silane according to Claim 1, characterized in that, in formula (2),
   - c = 0 or 1
   - d = 0
   - e = 1
   - Z is a phenyl group
   - R^2 are straight- or branched-chain C1-C4 and/or H alkyl groupings.

4. Silane according to Claim 1, characterized in that, in formula (2), Z is selected from: -O-, -CO-, -COO-, phenylene, -NR'- with R' = H or straight-chain or branched C1-C4, -S-alkyl.

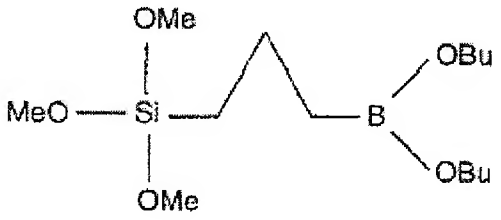
5. Silane according to any of claims 1 to 4, characterized in that, in formula (1), the radicals R^1, identical or different, are selected from:
   - C3-C10 straight-chain or branched alkyl and halogen alkyl radicals, preferably C1-C6,
   - C1-C10 cycloalkyl and halogen cycloalkyl radicals, preferably C5-C8
   - C2-C4 alkenyl radicals,
   - C6-C10 aryl and halogen aryl radicals.

6. Silane according to claim 5, characterized in that, in formula (1), the R^1 radicals, identical or different, are selected from methyl, phenyl, vinyl and trifluoro-3,3,3 propyl.

7. Silane in accordance with any of claims 1 to 6, characterized in that, in formula (1), the radical X is selected from halogen atoms, N-amino substituted, disubstituted N,N-amino, cetimonoxy, aldimonoxy, alkoxy, alkoxy alkylene-oxy, enoxy, acyloxy radicals.

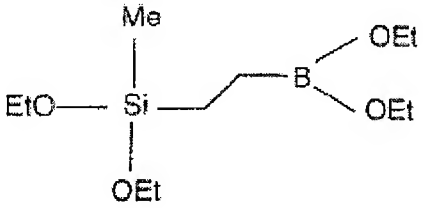
8. Silane according to claim 7, characterized in that the radical X is selected from the methoxy, ethoxy and acetoxy groupings.

9. Silane according to claim 2, having the formula:



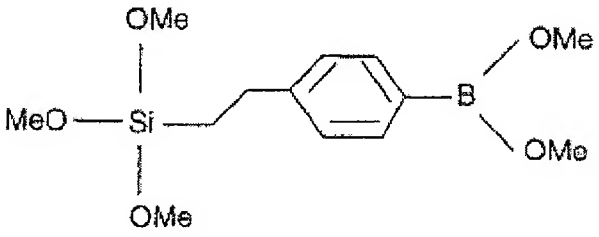
(A)

10. Silane according to claim 2, having the formula:



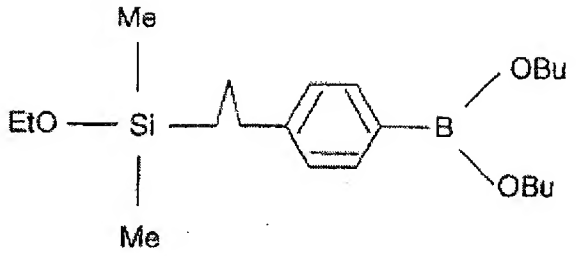
(B)

11. Silane according to claim 3, having the formula:



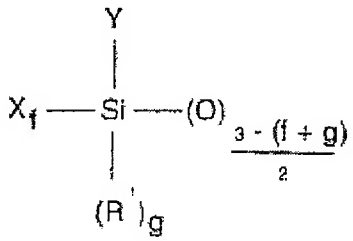
(C)

12. Silane according to claim 3, having the formula:



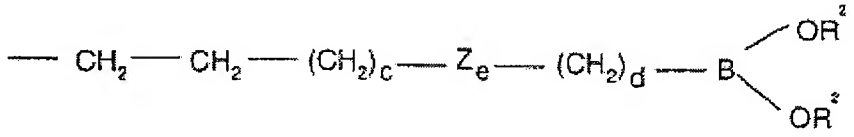
(D)

13. Polyorganosiloxane having per molecule at least one unit corresponding to the general formula (7):



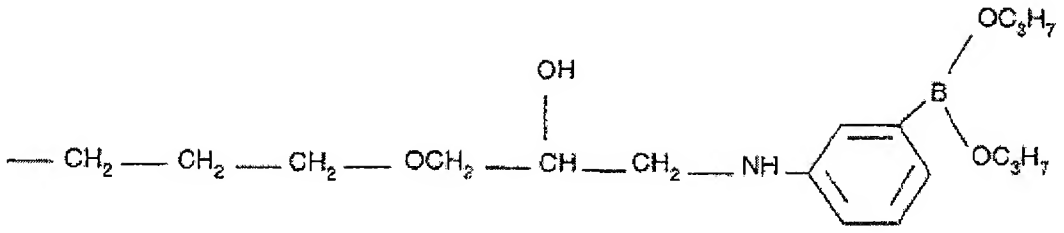
wherein:

- R^1 and X have the same meanings as claims 1 to 12,
- f is selected from among 0, 1 or 2,
- g is selected from among 0, 1 or 2,
- f + g is at most equal to 2,
- Y is a boronate group of formula (2)

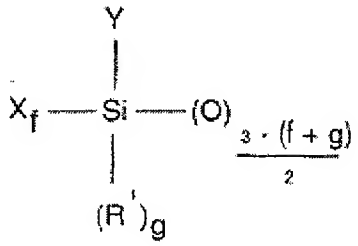


wherein:

c, Z, e, d and R^2 have the same meanings as claims 1 to 12, being excluded the polyorganosiloxane in which f + g = 0 and Y is such that:

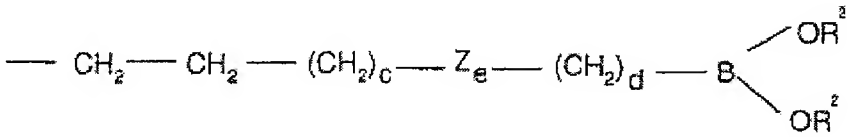


14. Polyorganosiloxane having per molecule, on the one hand, at least one unit corresponding to the general formula (7)



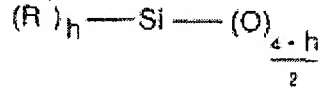
wherein:

- R^1 and X have the same meanings as claims 1 to 12,
- f is selected from among 0, 1 or 2,
- g is selected from among 0, 1 or 2,
- f + g is at most equal to 2,
- Y is a boronate group of formula (2)



wherein c, Z, e, d and R^2 have the same meanings as claims 1 without its disclaimer and 2 to 12,

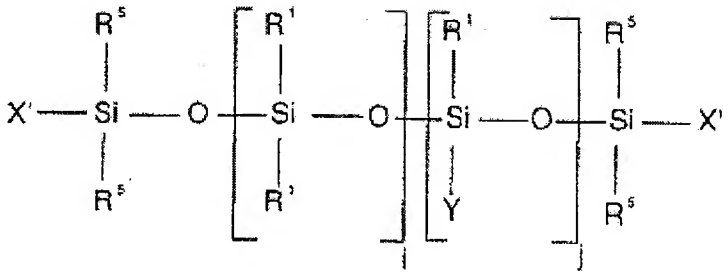
and, on the other, units corresponding to the formula (8)



wherein:

- R^1 has the same meaning as in claims 1 to 12,
- h is selected from 0, 1, 2 and 3.

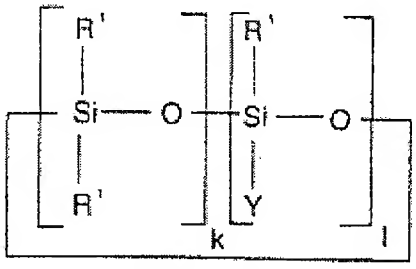
15. Polyorganosiloxane according to claim 13 without its disclaimer or 14, characterized in that it corresponds to formula (9)



wherein:

- R^1, X and Y have the same meanings as claims 13 without its disclaimer and 14,
- X' is selected from the radicals Y, R', hydroxyl and hydrogen atom,
- the radicals R^5, identical or different, are selected from the radicals R^1 and X,
- i is an integer between 0 and 1000,
- j is an integer between 0 and 50,
- if j = 0, at least 1 of the radicals X' is Y.

16. Polyorganosiloxane according to claim 13 without its disclaimer or 14, characterized in that it corresponds to formula (10)



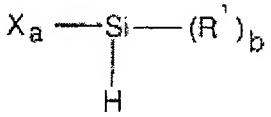
wherein:

- R^1 and Y have the same meanings as claims 13 without its disclaimer and 14,

- k is an integer between 0 and 9, inclusive,
- l is an integer between 1 and 9, inclusive,
- k + l is between 3 and 10, inclusive.

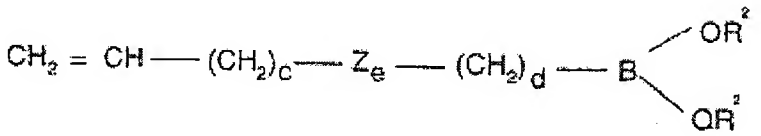
17. Process for the preparation of a silane according to claims 1 to 12, characterized in that a hydrosilylation reaction is carried out between:

- a silane of formula (3)



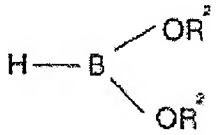
and

- an unsaturated alkyl dialkoxy borane of formula (4)

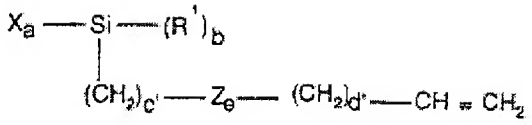


in which formulas (3) and (4) R^1, X, a, b, c, d, Z, e, R^2 have the same meanings as claims 1 to 12.

18. Process for the preparation of silanes according to claims 1 to 12, characterized in that a hydroboration reaction is carried out between:



(5)



(6)

wherein:

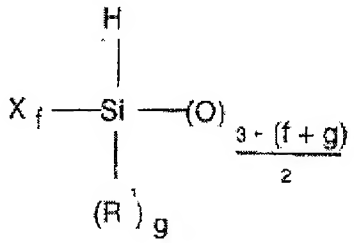
- c' = 2 to 18
- d' = 0 to 16
- c' + d' = 2 to 18

formulas (5) and (6) wherein R^2, X, a, R^1, b, Z have the same meanings as claims 1 to 12,

- with the possibility, where e = 0, that c' = 0 and d' = 1 (c' + d' = 0 or 1).

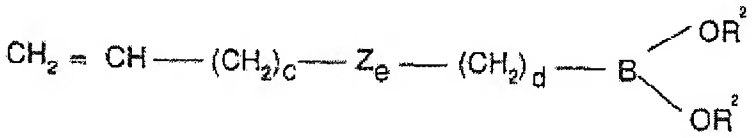
19. Process for the preparation of a polyorganosiloxane according to any one of claims 13 to 16, characterized in that a hydrosilylation reaction is carried out between:

- a polyorganosiloxane having per molecule at least unit of formula (11)



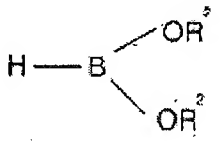
wherein R^1, g, X, f have the same meanings as claim 13 without its disclaimer or 14 and

- an unsaturated alkyl dialkoxy borane of formula (4)



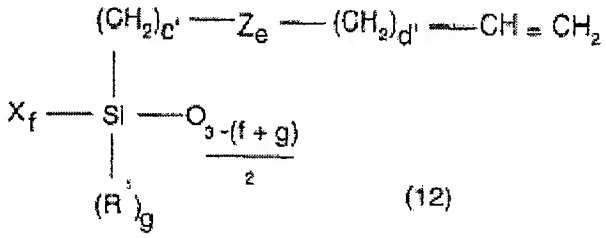
wherein R^2, c, Z, e, d have the same meanings as claims 1 without its disclaimer and 2 to 12.

20. Process for the preparation of a polyorganosiloxane according to any one of claims 6 to 9, characterized in that a hydroboration reaction is carried out by causing to react together:



(5)

and



(12)

wherein c' = 2 to 18

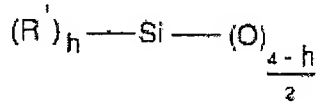
d' = 0 to 16

c' + d' = 2 to 18

in which formulas (5) and (12) R^2, X, f, R^1, g, Z and e have the same meanings as claim 13 without its disclaimer or 14,

- with the possibility, where e = 0, that c' = 0 and d' = 0 or 1 (c' +d' = 0 or 1).

21. Process for the preparation of a polyorganosiloxane according to any one of claims 13 without its disclaimer to 16, characterized in that hydrolysis and/or redistribution of a silane is carried out according to any one of claims 1 to 12 with a cyclic or straight-chain polysiloxane comprising the units of formula (8):



wherein:

- h = 2 or 3
- R^1 has the same meaning as in claims 1 to 12.

22. Process for the preparation of a polyorganosiloxane according to any one of claims 13 without its disclaimer to 17, characterized in that hydrolysis and/or redistribution of a silane is carried out according to any one of claims 1 to 12 with a hydrolysable silane of formula (13)

$$R^1_m X_n Si$$

wherein:

- m = 2 or 3
- m + n = 4
- X and R^1 have the same meanings as in claims 1 to 12.

23. Organopolysiloxane compound, storage-stable in the absence of humidity and capable of crosslinking through exposure to humidity in the absence of a crosslinking catalyst, comprising:

(A) - 100 parts by weight of at least one polydiorganosiloxane according to any one of claims 13 to 16,

(B) - from 0 to 250 parts by weight of a mineral filler.

24. Aqueous organopolysiloxane, storage-stable in the absence of humidity and capable of crosslinking through elimination of water, in the absence of a crosslinking catalyst, comprising:

(A) - 100 parts by weight of at least one polydiorganosiloxane according to any one of claims 13 to 16,

(B) - from 0 to 250 parts by weight of a mineral filler

(C) - from 0.5 to 20, preferably from 3 to 15, parts by weight of water

(D) - possibly a non-ionic, anionic, cationic or amphoteric tensioactive agent.

25. Method for conferring nonadherent and/or hydrophobic surface properties to a substrate, wherein a substrate, e.g. textile material, metal, stone or cement-based element, is coated

with a polyorganosiloxane according to any one of claims 13 to 16 or with a compound according to either of claims 23 and 24.

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ANNOUNCEMENT
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Process for the Synthesis of Substituted, Nitrogen-containing Heterocyclic Compounds

Submitters, inventors:

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Filing date: March 31, 1995

Debrecen, January 1995

The object of our invention is a new generally usable process for preparing substituted condensed, N-substituted azoles, possibly with homo- or heterocycles, containing at least two N atoms.

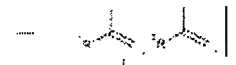
The azoles prepared according to our invention are biologically active substances that can be used as intermediates and/or as compounds with fungicidal, bactericidal, antithrombotic, inflammation-reducing, antiviral, or herbicidal effects (H. Vanden Bossche, W. Lauwers, G. Willemsens, P. Marichal, F. Cornelisson, and W. Cools. *Pestic. Sci.* **1984,** *13,* 188; G. I. Fiddler, P. Lumley, *Circulation* **1990,** *81* (Suppl. I), I 69; S. W. Wright, R. R. Harris, R. J. Collins, R. L. Corbett, A. M. Green, E. A. Wadman, and D. G. Batt, *J. Med. Chem.* **1992,** *35,* 3148; A. A. Umarov, S. S. Khalikov, M. Khaidarov, and L. A. Tyurina, *Uzh. Khim. Zh.* **1989,** *I* 40; *Chem. Abstr.* **1989,** *111,* 10920; Q. A. McKellar, and E. V. Scott, *J. Vet. Pharmacol. Ther.* **1990,** *13,* 223; S. Shirkura, A. Karasawa, and K. Kubo, *Arzneim.-Forsch.* **1991,** *42,* 1242; E. Nicolai, J. Goyard, T. Benchetrir, J. M. Teulon, F. Caussade, A. Virone, C. Delchambre, and A. Cloarec. *J. Med. Chem.* **1993,** *36,* 1175; J. A. Montgomery, S. J. Clayton, H. J. Thomas, W. M. Shannon, G. Arnett, A. J. Borner, T. K. Kion, G. L. Cantoni, and P. K. Chiang, *J. Med. Chem.* **1982,** *25,* 626; T. E. Spratt, and H. de los Santos, ***Biochemistry*** **1992,** *31,* 3688, German unexamined patent application 1,966,806, U.S. application 754,490, C.A. **1975**, *82,* 150,485).

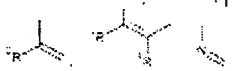
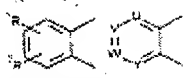
1-methyl-imidazole-5-carboxylates are key intermediates in the synthesis of physiologically active alkaloids, e.g., pylocarpin and its analogs (R. Karchlechner, M. Casutt, U. Neywang, and M. W. Schwarz, *Synthesis* **1994,** 247; J. M. Dener, L.-H. Zhang, and H. J. Rapoport, *J. Org. Chem.* **1993,** 58, 1169).

It is known (B. Testa and P. Jenner, *Drug Metab. Rev.* **1981,** *12(1),* 1-117 (p. 30); A. Wahhab, J. R. Smith, R. C. Ganter, D. M. Moore, J. Hondrelis, J. Matsoukas, and G. J. Moore, *Arzneim.-Forsch.* **1993,** *43,* 1157 (p. 1163)) that binding to cytochrome P-450 enzymes is significantly less blocked sterically in the case of azoles containing a nitrogen atom (e.g., 1,5-substituted imidazoles in contrast to 1,4-substituted imidazoles), and thus the biological effect is significantly stronger than in the case of less stable regioisomers. Cosar et al. compared the anti-trichomona or bactericidal effects of 5- and 4-nitro-1-alkyl imidazoles, and the 5-isomer turned out to be stronger in every case (C. Cosar, C. Crisan, R. Horclois, R. M. Jacob, J. Robert, S. Tchelitcheff, and R. Vaupre, *Arzeneimittel-Forsch.* **1966,** *16 (1),* 23). By proceeding according to the object of our invention, among other things, these and less stable isomers can be prepared advantageously.

The object of our invention is a process for preparing compounds of general formula 1,

where

the meaning of A is  the meaning of B is 

the meaning of D is  the meaning of BD is 

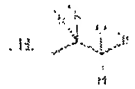
the meaning of R^1, R^2, and R^3 is H; a possibly substituted C_{1-4} alkyl; (substituted) phenyl; $NHCOC_{1-4}$ alkyl; $COOC_{1-4}$ alkyl

the meaning of U, V, W, Y, and Z is CH; N; CO; CS, N–C_{1-8}alkyl; C–OC_{1-4} alkyl; C–SC_{1-4} alkyl; C–$N(C_{1-4}$ alkyl$)_2$

the meaning of n is 0, 1

the meaning of X is a chlorine, bromine, or iodine atom; C_{1-4} alkyl–SO_2; OSO_3R^7 C_{1-3} fluoridated alkyl–SO_3, (substituted) phenyl–SO_3

the meaning of R^7 is –; possibly substituted C_{1-8} alkyl; N-containing heteroaryl

the meaning of R^8 is 

the meaning of R^4, R^5, and R^6 is H; alkyl; cycloalkyl; Q

the meaning of Q is CN; $COOC_{1-4}$ alkyl; COC_{1-4} alkyl; CO (substituted) phenyl; SO_2C_{1-4} alkyl; SO_2 (substituted) phenyl

such that

a.) the azoles characterized by general formula 2 containing an NH,

where the meaning of A, B, and D is as above, are given

with the α,β-unsaturated compounds characterized by general formula 3

where the meaning of R^4, R^5, and R^6, is as above,

with the amidine of general formula 4, which functions as a base and/or a transfer reagent,

where the meaning of E, J, and L is –; H; an aliphatic ring residue; an N-containing aliphatic ring residue,

the N-(substituted) ethylene derivative of general formula 5, forming a sub-case of formula 1.

where the meaning of A, B, D, R^4, R^5, R^6, and Q is as above,

b.) The azoles of general formula 5 are converted to a quarternary salt with the alkylizing agent of general formula 6,

where the meaning of X is as above,

then the (substituted) ethylene group is taken up selectively with a base, with the less preferred alkyl azoles of general formula 1 being removed in a Hofmann-type decomposition.

Proceeding according to point a.) of our process, the five-member N-containing heterocyclic compound of general formula 2 or its ring-condensed derivative is made to react at 0-150 °C in the presence of an organic basic catalyst of the amidine type of general formula 4, a (substituted) guanidine base, appropriately 1,5,7-triazabicyclo-[4,4,0]-dec-5-ene (TBD), its 7-methyl derivative (7-Me-TBD) , or a variant

applied to a polymer carrier (TBD-P) in a polar aprotic solvent, e.g., acetonitrile, nitromethane, acetone, dimethyl sulfoxide, N,N-dimethyl formamide, N,N-dimethyl acetamide, N-methyl-2-pyrrolidone, or a mixture thereof, advantageously acetonitrile, with 1-10 mole equivalents of an α,β-unsaturated compound of general formula 3.

- the reaction mixture – filtered in the case where a catalyst applied to a carrier is used – is evaporated,

- treated with water or an aqueous solution of an organic salt, advantageously ammonium chloride or ammonium carbonate; the product of general formula 5 forming the sub-case of formula 1, is isolated by filtering.

Proceeding according to point **b.)** of our process,

- the reaction mixture containing the product characterized by general formula 5 prepared according to point a.),

- either taking the product of general formula 5, prepared according to point a.), up in a polar solution, such as nitromethane, alcohols, dimethyl sulfoxide, N,N-dimethyl formamide, N,N-dimethyl acetamide, N-methyl 2-pyrrolidone, advantageously acetonitrile,

adding 0.001-1 mole equivalents of a halide-ion catalyst, advantageously an alkaline iodide, to the solution obtained, it is made to react with 0.9-10 mole equivalents of the alkylizing agent of general formula 6 at 0-150 °C.

- the reaction mixture is evaporated, the raw azolium salt obtained is dissolved in water, and by washing the aqueous phase with a water-immiscible solvent, an aqueous solution of the compound of general formula 7 is obtained, representing the sub-case of general formula 1.

- or the product of general formula 7 representing the sub-case of general formula 1 is obtained by adding an aprotic solvent, such as ether, acetone, or ethyl acetate, to the reaction mixture obtained and isolated by filtering,

then, to:

- the azolium salt of general formula 7,

- the reaction mixture containing it,

- or an aqueous solution

is added to the [compound] representing the sub-case of general formula 1.

0.95-5 equivalents of base, advantageously an alkali alcohol, alkali hydroxide, alkali carbonate, alkali hydrogen carbonate, or amine derivative or an alcohol and/or water solution thereof is added and stirred at 0-100 °C.

- cooled, and by treating it with water or a 10-30 % solution and/or an ammonium salt, advantageously ammonium chloride or ammonium carbonate, the separated product of general formula 1 ($n = 0$, $R^x = -$) is isolated by filtering,

- the reaction mixture obtained is stirred with some adsorbent, advantageously silica gel, aluminum oxide, purifying carbon, or a mixture thereof, and filtered, and the product of general formula I [sic]($n = 0$, $R^x = -$) is obtained by evaporating the filtrate,

- or, to the extent that it contains an organic solvent, the solvent is removed from the raw reaction mixture in vacuum, the residue is taken up into water, the water-immiscible solvent is extracted, and the organic phase is purified, dried, and evaporated.

According to the literature, azoles of general formula 2, with direct alkylization with an alkylizing agent, generally lead to mixtures in which the various regioisomers (where this is possible) and the N,N'-dialkyl quaternary azolium salts are equally present. Although they can be separated in some rare cases, in cases where regioisomers can form, the more preferred regioisomer is always formed mostly with direct alkylization.

Processes are known for separating the less preferred isomers from azoles containing a free NH group, in which the preferred N atom is protected with a protecting group during direct alkylization and the protecting group is finally split off. Thus after histidine and its derivatives are benzylized, then after the alkylization, the protecting benzyl group is hydrogenolized on a palladium catalyst (P. Sauerberg, J. Chen, E. WoldeMussie, and H. Rapoport, *J. Med. Chem.* **1989,** *32,* 1322). To synthesize trialkyl-9-methyl xanthine derivatives, 7-benzyl or 7-methoxymethyl xanthines have been used (H. G. von Schuh, German patent 1,113,696, *C.A.* **1962,** 56, 12, 909). In the case of nitroimidazoles, the acetoxymethylene protecting group can be removed by boiling in an aqueous medium (C. Bonnamas, V. Massonneau, M. Mulhauser, and N. Rouy, European patent application 325,512 (July 26, 1989); *Chem. Abstr.* **1990,** *112,* 77185). In the case of (4-substituted) imidazoles, 1,2,4-triazole, and benzotriazole, an acyl group can likewise be removed by hydrolysis (R. A. Olofson and R. V. Kendall, *J. Org. Chem.* **1970,** *35,* 2246; C. Kashima, Y. Harada, and A. Hosomi, *Heterocycles* **1993,** *35,* 433; T. Kamijo, R. Yamamoto, H. Harada, and K. Iizuka, *Chem. Pharm. Bull.,* **1983,** *31(4), 1213).* In the case of urocanic acid (imidazole-4-acrylic acid), phenacyl can be removed with the acetic-acid/zinc system (N. Lauth-de Viguerie, N. Sergueeva, M. Damiot, H. Mawlawi, M. Riviere, and A. Lattes, *Heterocycles* **1994,** *37,* 1561).

The alkyl-type protective groups (benzyl, phenacyl, acyloxymethylene) have the common disadvantage that they are not taken up regioselectively: the protected azole can be obtained only with a low yield and purity. Using an acyl (acetyl, ethoxycarbonyl, benzyl) protective group is more advantageous from this viewpoint; they are said to be taken up regioselectively. However, the protected azoles obtained in this way, because of their characteristic of strong electron absorption, can only be removed by using alkylizing agents that are very active and difficult to prepare (trialkyl oxonium tetrafluoroborates) or can be quaternized [only] at a high temperature and pressure (up to 7000 bars). It is also known that the acylized azoles compounds are difficult to store and decompose due to the effect of moisture.

According to previous literature or our own experience, because of regioselectivity, in the case of azoles of general formula 2, the cyanoethyl group is a special alkylizing agent.

Imidazoles have been made to react thermically with (substituted) acrylonitrile (N. Sawa and S. Okamura, *Nippon Kagaku Zasshi* **1969,** *90(7),* 704; *Chem. Abstr.* **1969,** *71,* 10173. M. Yamauchi and M. Masui, *Chem. Pharm. Bull.* **1976,** *24(7),* 1480. W. B. Wright, J. B. Press, US patent *4,619,941* (October 28, 1986); *Chem. Abstr.* **1987,** *106,* 102285).

4-aryl imidazole has been cyanoethylized with a basic catalyst in ethanol in the presence of potassium hydroxide (M. A. Iradyan, A. G. Torsyan, R. G. Mirzoyan, I. P. Badalyants, Z. S. Isaakyan, D. Sh. Manucharyan, M. Kh. Dayan, G. S. Sakanyan, I. A. Dzagatspanyan, N. E. Akonayn,

Y. Xh. Ter-Zaharyan, and A. A. Aroyan, *Khim-Pharm. Zh.* **1977,** *11,* 42; *Chem. Abstr.* **1978,** *88,* 22759y, or with quaternary ammonium-hydroxide catalysts, in various solvents – primarily dioxane, such as 4-nitroimidazole with benzyl-triethyl/trimethyl ammonium hydroxide (C. Cosar, C. Crisan, R. Horclois, R. M. Jacob, J. Robert, S. Tchelitcheff, and R. Vaupre, *Arzneimittel-Forsch.* **1966,** *16(1),* 23), benzoimidazole (J. Diamond, and R. A. Wohl, *European patent application 34,116* (August 19, 1981); *Chem. Abstr.* **1981,** *95,* 203961), 5-nitro and 2-methyl-5-nitrobenzoimidazole (A. M. Efros, *Zhur. Obsh. Khim.* **1960,** *30,*3565; *Chem. Abstr.* **1961,** *55,* 18712d).

Imidazole has been made to react with phenylvinyl ketone with a hydroxide catalyst (S. V. Bogatkov, B. M. Kormanskaya, V. Mochalin, and E. M. Cherkasova, *Khim. Geterotsikl. Soedin.* **1971,** *7(5),* 662-4; *Chem. Abstr.* **1972,** *76,* 59525).

Under various conditions, Michael additions of 1,2,3-triazole or benzotriazole have been investigated with a catalyst of benzyl-trimethyl ammonium hydroxide or pyridine (R. H. Wiley, N. R. Smith, D. M. Johnson, and J. Moffat, *J. Am. Chem. Soc.* **1954,** 76, 4933). The reaction of 2-methyl 4-nitroimidazole with 5 kinds of Michael acceptors with numerous catalysts in various solvents [has been studied], and the pyridine /dimethyl-sulfoxide system has been found to be the best. Under such conditions, acrylonitrile was made to react at 135 °C for 10 hours (A. K. S. B. Rao, C. G. Rao, and B. B. Singh, *J. Org. Chem.***1990,** *55,* 3702). Catalysis with other tertiary amines has been described in the reaction of imidazole and methyl acrylate in the presence of triethylamine [has been studied] (S. V. Bogatkov, B. M. Kormanskaya, V. Mochalin, and E. M. Cherkasova, *Khim. Geterotsikl. Soedin.* **1971,** *7(5), 662-4; Chem. Abstr.* **1972,** *76,* 59525). Derivatives of xanthine of natural origin [have been studied], such as theophillin with acrylonitrile in the presence of copper with a catalyst of sodium hydroxide or benzyl-trimethyl ammonium hydroxide (K. Doebel and H. Spiegelberg. US patent 2,761,862 (1956); *Chem. Abstr.* **1957,** *51,* 3676; A. Rybar and L. Stibrányi, *Collect. Czech Chem. Commun.* **1973,** *38(5),* 1571). Theophillin and theobromine have been made to react with the reagents acrylonitrile, acrylic acid, and ethyl acrylate in the presence of benzyl-trimethyl ammonium hydroxide (R. Zelnik and M. Pesson, *Bull. Chem. Soc. Fr.* **1959,** 1667).

These processes generally require high temperatures and long reaction times, which lead to a destruction of regioselectivity and to side reactions (e.g., polymerization of the Michael acceptor, addition of components of the non-azole type in the system). The side reactions and the use of solvents with high boiling points make it difficult to obtain the product from the reaction mixture, reduce the yield, and ruin the quality of the product.

A cyanoethyl protecting group has also been used in the alkylization of imidazole and benzoimidazole (A. Horváth, *Synthesis,* **1994,** 104), but the process given is complex, and requires a processing step associated with large loss of materials (acidic re-solution and extraction, realkalinization, reextraction). Regioselectivity was not investigated at that time. Some of the bases used (sodium hydroxide, sodium alcoholate) cannot be used in the case of azoles substituted with strongly electron-absorbing groups (for example, nitroimidazoles), where from the quaternary salt of N-alkyl-N'-cyanoethyl azolium, splitting of the alkyl group, not the cyanoethyl group is preferred, or in the case of molecules sensitive to nucleophilic bases (for example, the phthalimide ring opens due to the effect of these bases).

The advantage of the process according to our invention is that, on the one hand, azoles of formula 2 containing an NH group and ring-condensed derivatives can be made to react as a base and/or transfer reagent that causes a strong, non-nucleophilic Michael addition in the presence of an amidine or guanidine of general formula 4, which makes a more stable compound of general formula 3, the Michael acceptor forming the sub-case of general formula 1, possible by a fast reaction under mild conditions, at a low temperature (mostly at room temperature), with a high yield, practically without side reactions, with regioselectivity favored kinetically. It can be used as well as an intermediary in the conversion of the Q-electron-absorbing functional group – to give N-(substituted) ethylene derivatives that are Michael adducts that can be prepared with high yield and purity by simple methods. The practically fully selective conversion makes it possible in many cases that, in the case of further alkylization, the Michael adducts cannot be isolated from the reaction mixture.

On the other hand, the process according to our invention makes it possible to use the Michael adducts as N-protected azoles in a position preferred for the production of less preferred N-substituted regioisomers: by alkylizing the N,N'-substituted azolium salts of general formula 7 obtained with alkylizing agents – isolating them, if necessary, compounds are usually obtained that can be isolated as crystals and can be expected to have other advantageous characteristics – *in situ,* under conditions of a Hofmann-type decomposition, by splitting the (substituted) ethyl group selectively; the product obtained is a (less preferred) N-substituted derivative of the starting azole. Both the quaternization and decomposition of the quaternary salt can be performed practically quantitatively, under mild conditions, and the final products are obtained regioselectively, with high yield and high purity.

Using a cyanoethyl or substituted derivative as a protective group is especially advantageous in the cases of alkylization when the products of general formula 1 are slightly soluble in water and the quaternary salts are obtained by filtering from the decyanoethylization reaction mixture. Using a 2-(alkoxycarbonyl) ethyl protective group is advantageous in the case of final products of general formula 1 that are soluble in water, because when the protective group is split off, a β-substituted propionic-acid salt is formed that dissolves well in water, but poorly in organic solvents, and does not contaminate the products extracted into the organic solvent.

To synthesize the less preferred N-substituted regioisomers of azoles, C-substituted with strongly groups with a electron-absorbing effect, containing a free NH group, it is advantageous to use ethyl protective groups substituted in the 2-position with a keto or sulfonyl group, which make it possible for them to be removed with weak, non-nucleophilic bases, thus decomposition of quaternary azolium salts containing a strongly election-absorbing group or groups sensitive to nucleophiles (ring opening, aromatic nucleophilic substitution) can be performed without side reactions.

Details of our process will be presented in the following examples, without limiting our invention to them.

Examples

1.) 4-phenyl-1H-imidazole-1-propionic-acid nitrile

4.32 g 4-phenyl imidazole, 3.6 ml acrylonitrile, and 0.14 g TBD are stirred in 10 ml acetonitrile for 10 minutes, a solution of ammonium chloride is added to the residue obtained by evaporation, it is cooled and stirred, filtered, washed, and dried. Yield. 5.65 g (95 %), m.p.: 114-115.5 °C.

2.) 4-nitro-1H-imidazole-1-propionic-acid nitrile

5.65 g 4-nitroimidazole, 5 ml acrylonitrile, and 0.28 g TBD are stirred in 15 ml DMSO at 100 °C for 5 hours. It is processed by evaporation similar to example 1. Yield: 7.8 g (94 %). By recrystallizing from ethyl acetate, m.p.: 112-113 °C.

3.) α,4-dimethyl-1H-benzoimidazole-1-propionic-acid nitrile

2.64 g 4-methyl benzoimidazole, 1.67 g crotonic-acid nitrile, and 0.14 g TBD are stirred in 10 ml acetonitrile at 50 °C for 1 hour. It is evaporated and processed in the manner of the previous example. Yield: 3.82 g (96 %), m.p.: 117-118 °C. ^{1}H NMR ($CDCl_3$): 1.89 (d, 3H, J= 7.1), 2.69 (s, 3H), 2.88-2.98 (m, 2H), 4.83 (sext, 1H, J= 7.1), 7.10-7.17 (m, 1H), 7.18-7.31 (m, 2H), 8.03 (s, 1H). MS(EI^+, 70 eV): m/z (%): 199 (M^+, 28). 159 (100), 131 (15), 77 (161).

4.) 1H-1,2,4-triazole-1-propionic-acid nitrile

34.52 [g] 1,2,4-triazole, 50 ml acrylonitrile, and 0.7 g TBD are taken up into 50 ml acetonitrile, stirred for 4 hours, and evaporated. Yield: 65.16 g, m.p.: 36-37 °C (hexane-EtOAc). [^{1}H] NMR ($CDCl_3$): 3.00 (t, 2H). 4.47 (t, 2H). 8.01 (s, 1H). 8.23 (s, 1H).

5.) 1H-1,2,4-triazole-1-propionic-acid ethyl ester

6.9 g 1,2,4-triazole, 12 g acrylic-acid ethyl ester, and 0.28 g TBD are taken up into 20 ml acetonitrile, stirred for 5 hours, then evaporated. The raw product is purified by eluting with a 100:5 chloroform:methanol mixture. 15.8 g (93 %) of oil is obtained. ^{1}H NMR ($CDCl_3$): 1.23 (t, 3H), 2.91 (t, 2H), 4.14 (q, 2H), 4.48 (t, 2H), 7.93 (s, 1H), 8.16 (s, 1H).

6.) 3-(4-methyl-1H-imidazole-1-yl)-pentane-dicarbonic-acid diethyl ester

8.2 g 4-methyl imidazole, 18.6 g diethyl gutaconate, and 0.7 g TBD in 20 ml acetonitrile are allowed to stand for 40 days, then evaporated. The raw product is clarified from a dilute solution of hydrochloric acid. Its germicidal effect is set to pH 8 with ammonia solution, then it is extracted with dichloromethane. The organic phase is dried and evaporated. Yield: 18.2 g (68 %).

7.) 1-(phenylmethyl)-5-methyl 1H-imidazole

2.68 g of the product obtained according to example 6 and 1.88 g benzyl bromide in 5 ml acetonitrile were boiled under reflux cooling for 3 hours and evaporated. 10 ml of a 2-M ethanol solution of NaOEt is added, evaporated after 10 minutes of stirring, acidified with cold dilute hydrochloric acid, and washed with ether. The aqueous phase was clarified at room temperature, set to pH 8 with ammonia solution, cooled, dried, and

washed with water. Yield: 1.04, m.p.: 107-109.5 °C (hexane ether). ^{1}H NMR ($CDCl_3$): 2.08 (s, 3H), 5.04 (s, 2H), 6.82 (m, 1H), 6.98-7.10 (m, 2H), 7.22-7.40 (m, 3H), 7.46 (m, 1H).

8.) 1-(2-cyanoethyl)-4-phenyl-3-methyl-1H-imidazolium bromide

4.61 g 4-phenyl imidazole, 2.6 ml acrylonitrile, and 1 g TBD-P are stirred at room temperature in 20 ml acetonitrile for 130 hours and filtered. 4.3 ml methyl iodide is added to the filtrate. It is boiled for 6 hours under reflux cooling, cooled, filtered, washed with acetone, and dried. Yield: 9.0 g (83 %), m.p.: 179.5-180.5 °C (acetonitrile). ^{1}H NMR (DMSO-d_6): 3.27 (t, 2H), 3.88 (s, 3H), 4.56 (t, 2H), 7.60 (s, 5H), 8.06 (m, 1H), 9.34 (m, 1H).

9.) 1-(2-cyanoethyl)-3-(cyanopropyl)-4-phenyl-1H-imidazolium bromide

1.97 g of the product prepared according to example 1.), 1.48 g 4-bromobutyronitrile, and 0.015 g NaI are boiled in nitromethane under reflux cooling, cooled, diluted with 20 ml ether, filtered, washed with ether, and dried. Yield: 3.21 g (93 %), m.p.: 131-132 °C (MeCN). ^{1}H NMR (DMSO-d_6): 1.99 (quint, 2H, J= 7.2), 2.58 (t, 2H, J= 6.7), 3.33 (t, 2H, J= 6.2), 4.33 (t, 2H, J= 7.2), 4.60 (t, 2H, J= 6.2), 7.60 (s, 5H), 8.11 (d, 1H, J= 1.5), 9.60 (d, 1H, J=1.5).

10.) 1-(2-cyano-1-methylethyl)-3-(3-cyanopropyl)-4-methyl-1H-benzoimidazolium bromide

Proceeding according to example 9 from 1.99 g of the product of example 3.) and 1.48 g 4-bromobutyronitrile. Yield: 32.6 g (94 %), m.p.: 187-188 °C (nitromethane). ^{1}H NMR (DMSO=d_6): 1.78 (d, 3H, J= 6.6), 2.30 (quint, 2H, J= 6.5), 2.77 (t, 2H, J= 6.5), 2.81 (s, 3H), 3.40 (d, 2H, J= 6.0), 4.74 (t, 2H, J= 6.5), 5.44 (sext, 1H, J= 6.0), 7.49 (d, 1H, J= 7.0), 7.61 (t, 1H, J= 7.0), 8.07 (d, 1H, J= 7.0), 10.13 (s, 1H).

11.) 1-(2-cyanoethyl)-4-(3-cyanopropyl)-1H-1,2,4-triazolium bromide

Proceeding according to example 9 from 1.22 g 1H-1,2,4-triazole-1-propionic-acid nitrile. Yield: 20.5 g (76 %), m.p.: 104-105.5 °C. ^{1}H NMR (DMSO-d_6): 2.20 (quint, 2H), 2.66 (t. 2H), 3.21 (t, 2H), 4.39 (t. 2H), 4.63 (t, 2H), 9.35 (s, 1H), 10.28 (s, 1H).

12.) 1-(2-cyanoethyl)-3-(phenylmethyl)-4-phenyl-1H-imidazolium bromide

1.97 g of the product of example 1 and 1.71 g benzyl bromide in 5 ml acetonitrile are boiled for 50 hours under reflux cooling, diluted with ether, and filtered. Yield: 3.49 g (95 %), m.p.: 173-174 °C (acetonitrile). ^{1}H NMR (DMSO-d_6): 3.30 (t, 2H, J= 6.1), 4.60 (t, 2H, J= 6.1), 5.55 (s, 2H), 7.05-7.14 (m, 2H), 7.31 (m, 3H), 7.50 (m, 5H), 8.10 (d, 1H, J= 1.3), 9.47 (d, 1H, J= 1.3).

13.) 1-(2-cyano-1-methylethyl)-3-(phenylmethyl)-4-methyl-1H-benzoimidazolium bromide

Proceeding according to point 12.) from 1.99 g of the product of point 3.) and 1.71 g benzyl bromide (reaction time: 20 hours). [Yield:] 3.55 g, m.p.: 214-215 °C (acetonitrile). ^{1}H NMR (DMSO-d_6): 1.85 (d, 3H, J= 6.7), 2.49 (s, 3H), 3.50 (d, 2H, J= 6.2), 5.51 (sext, 1H, J= 6.2), 6.01 (s, 2H), 7.18-7.30 (m, 2H), 7.35-7.50 (m, 4H), 7.62 (t, 1H, J= 7.8), 8.13 (d, 1H, J= 7.8), 10.30 (s, 1H).

14.) 1-(2-cyanoethyl)-4-(phenylmethyl) 1H-1,2,4-triazolium bromide

Proceeding according to point 12.) from 1.22 g 1H-1,2,4-triazole-1-propionic-acid nitrile and 1.71 g benzyl bromide. Yield: 2.38 g (81 %), m.p. 166.5-168 °C. ^{1}H NMR (DMSO-d_6): 3.23 (t, 2H), 4.71 (t, 2H), 5.57 (s, 2H), 7.40-7.54 (m, 5H), 9.43 (s, 1H), 10.28 (s, 1H).

15.) 1-(2-(ethyoxycarbonyl)ethyl-4-(phenylmethyl) 1H-1,2,4-triazolium bromide

Proceeding according to point 12.) from 4.23 g of the product of point 5.) and 4.28 g benzyl bromide. Yield: 7.28 g (85 %), m.p.: 116-117 °C. ^{1}H NMR (DMSO-d_6): 1.17 (t, 3H), 3.05 (t, 2H), 4.10 (q. 2H), 4.67 (t, 2H), 5.63 (s, 2H), 7.42-7.61 (m, 5H), 9.46 (s, 1H), 10.42 (s, 1H).

16.) 1-(2-cyano-1-methylethyl)-3-(2-propenyl)-4 methyl-1H-benzoimidazolium bromide

Proceeding according to point 12 from 1.99 g of the product of point 3.) and 1.3 g allyl bromide. Yield: 2.88 g (90 %), m.p.: 180-182 °C (acetonitrile). ^{1}H NMR (DMSO-d_6): 1.78 (d, 3H, J= 7.1), 2.74 (s, 3H), 3.42 (d, 2H, J= 7.1), 5.01 (m, 1H), 5.30-5.54 (m, 2H + 1H), 6.16-6.40 (m, 1H), 7.47 (d, 1H, J= 7.5), 7.61 (t, 1H, J= 7.5), 8.07 (d, 1H, J= 7.5), 10.06 (s, 1H).

17.) 1-(2-cyanoethyl)-3-(((((4-methylcarbonyl)phenyl)amino)carbonyl)methyl)-4-phenyl-1H-imidazolium bromide

1.97 g of the product of point 1.) and 2.72 g 2-bromo-N-((4-methoxycarbonyl)phenyl)acetamide in 10 ml acetonitrile are boiled under reflux heating, cooled, diluted with acetonitrile, filtered, washed, and dried. Yield: 4.41 g (94 %), m.p.: 205-207 °C (MeOH). ^{1}H NMR (DMSO-d_6): 3.32 (t, 2H, J= 6.9), 3.82 (s, 3H), 4.68 (t, 3H, J=6.9), 5.28 (s, 2H), 7.55-7.60 (m, 5H), 7.63 (m, 2H), 7.94 (m, 2H), 8.11 (d, 1H, J= 1.2), 9.58 (d, 1H, J= 1.2), 10.84 (s, NH).

18.) 1-(2-cyano-1-methylethyl)-3-(((((4-methoxycarbonyl)phenyl)amino)carbonyl)methyl)-4-methyl-1H-benzoimidazolium bromide

Proceeding according to point 17.) from 1.99 g of the product of point 3.) and 2.72 g 2-bromo-N-((4-methoxycarbonyl)phenyl) acetamide. [Yield:] 4.48 g (95 %), m.p.: 141-143 °C (MeOH). ^{1}H NMR (DMSO-d_6): 1.39 (d, 3H, J= 7.2), 2.67 (s, 3H), 3.43 (d, 2H, J= 7.2), 3.82 (s, 3H) 5.49 (sext, 1H, J= 7.2), 5.72 (s, 2H), 7.48 (d, 1H J= 7.1), 7.64 (t, 1H, J= 7.1), 7.76 (m, 2H), 7.98 (m, 2H), 8.10 (d, 1H, J= 7.1), 10.05 (s, 1H), 11.17 (s, NH).

19.) 1-(2-cyanoethyl)-4-(((((4-methoxycarbonyl)phenyl)amino)carbonyl)methyl)-1H-1,2,4-triazolium bromide

Proceeding according to point 17.) from 1.22 g 1H-1,2,4-triazole 1-propanoic-acid nitrile and 2.72 g 2-bromo-N-((4.-methoxycarbonyl)phenyl) acetamide (1 hour). [Yield:] 3.66 g (93 %), m.p.: 227-228 °C (Me OH). ^{1}H NMR (DMSO-d_6): 3.28 (t, 2H), 3.84 (s, 3H), 4.83 (t, 2H), 5.42 (s, 2H), 7.74 (d, 2H), 7.98 (d, 2H), 9.30 (s, 1H), 10.22 (s, 1H), 11.01 (s, NH).

20.) 4-(2-cyanoethyl)-1-(((((4-methoxycarbonyl)phenyl)amino)carbonyl)methyl)-1H-1,2,4-triazolium bromide

Proceeding according to point 17 from 0.30 g 4H-1,2,4-triazole-4-propionic-acid nitrile and 0.67 g 2-bromo-N-((4-methoxycarbonyl)phenyl)acetamide. Yield: 0.41 g (42 %), m.p.: 206-208 °C (MeOH).

^{1}H NMR (DMSO-d_6): 3.32 (t, 2H), 3.84 (s, 3H), 4.73 (t, 2H), 5.59 (s, 2H), 7.74 (d, 2H), 7.97 (d, 2H), 9.41 (s, 1H), 10.34 (s, 1H), 11.10 (s, NH)

21.) 5-phenyl-N-((4-methoxycarbonyl)phenyl)-1H-imidazole 1-acetamide

2.35 g of the product of point 17.) in 5 ml of a 2-M methanol solution of NaOMe, is stirred for 5 minutes at room temperature, cooled, and treated with an aqueous solution of ammonium chloride, stirred for 2 hours, filtered, washed with water then cold acetone, and dried. Yield: 1.61 g (96 %), m.p.: 220-222 °C (methanol/water). ^{1}H NMR (DMSO-d_6): 3.89 (s, 3H), 4.82 (s, 2H), 7.18 (d, 1H, J= 1.1), 7.30-7.45 (m, 5H), 7.51 (m, 2H), 7.67 (d, 1H, J= 1.1), 7.99 (m, 2H + NH).

22.) 7-methyl-N-((4-methoxycarbonyl)phenyl 1H-benzoimidazole-1-acetamide

Treating 2.36 g of the product of point 18.) according to point 21.). Yield: 1.57 g (97 %), m.p.: 242-244 °C (MeOH/water). ^{1}H NMR (DMSO-d_6): 2.65 (s, 3H), 3.88 (s, 3H), 5.15 (s, 2H), 7.08 (d, 1H, J= 7.4), 7.19 (t, 1H, J= 7.4), 7.49-7.68 (m, 2H + 1H), 7.96 (m, 2H), 8.61 (s, NH).

23.) N-((4-methoxycarbonyl)phenyl) 4H-1,2,4-triazole 4-acetamide

Treating 1.97 g of the product of point 19.) according to point 21.) Yield: 1.19 g (91 %), m.p.: 280-282 °C (MeOH). ^{1}H NMR (DMSO-d_6): 3.82 (s, 3H), 5.07 (s, 2H), 7.72 (d, 2H), 7.95 (d, 2H), 8.49 (s, 2H), 10.74 (s, NH).

24. N-((4-methoxycarbonyl)phenyl)-1H-1,2,4-triazole 1-acetamide

Based on point 21.) from 0.4 g of the product of point 20.). Yield: 0.22 g (85 %), m.p.: 218-220 °C (MeOH). ^{1}H NMR (DMSO-d_6): 3.83 (s, 3H), 5.19 (s, 2H), 7.72 (d, 2H), 7.95 (d, 2H), 8.01 (s, 1H), 8.56 (s, 1H), 10.76 (s, NH).

25. 5-phenyl-1-methyl 1H-imidazole

8 g of the product of point 8.) in 8 ml of a 20% sodium-hydroxide solution is stirred at room temperature for 1 hour, cooled, filtered, washed, and dried. Yield: 3.61 g (97 %), m.p.: 90.5-93 °C.

26. 5-phenyl-1-(phenylmethyl) 1H-imidazole

1.84 g of the product of point 12.) is treated according to point 25.). Yield: 1.05 g (90 %), m.p.: 115-117 °C. ^{1}H NMR ($CDCl_3$): 5.15 (s, 2H), 6.96-7.07 (m, 2H), 7.14 (d, 1H, J= 0.9), 7.24-7.42 (m, 8H), 7.57 (d, 1H, J= 0.9).

27.) 1-(phenylmethyl)-7-methyl 1H-benzoimidazole

1.85 g of the product of point 13.) is treated according to point 25.). Yield: 0.98 g (88 %), m.p.: 159-160 °C. ^{1}H NMR ($CDCl_3$): 2.47 (s, 3H), 5.67 (s, 2H), 6.92-7.05 (m, 3H), 7.18 (t, 1H, J= 8.2), 7.25-7.39 (m, 3H), 7.70 (d, 1H, J- 8.2), 7.87 (s, 1H).

28.) 5-phenyl-1H-imidazole-1-butanoic-acid nitrile

Proceeding according to point 9.), the reaction mixture obtained is evaporated, and the quaternary salt is stirred with 10 ml of a 20% sodium-lye solution at room temperature for 1 hour and extracted with ethyl acetate, and the organic phase

is extracted with 20 ml of 1-N solution of HCl. The aqueous phase is clarified, and the pH is set to between 8 and 9 with an ammonia solution and extracted 3 times with 20 ml dichloromethane, and the organic phase is dried and evaporated. Yield: 1.96 g (93 %), m.p.: 58-59 °C. ^{1}H NMR ($CDCl_3$): 1.88 (quint, 2H, J= 7.0), 2.17 (t, 2H, J= 7.0), 4.19 (t, 2H, J= 6.9), 7.10 (d, 1H, J= 1.0), 7.30-7.52 (m, 5H), 7.60 (d, 1H, J= 1.0).

29.) 7-methyl-1H-benzoimidazole-1-butanoic-acid nitrile

By making the reaction mixture containing the quaternary salt obtained according to point 10.) react according to point 28.) and processing, the yield is 1.89 g (95 % with respect to the product according to 3.)). ^{1}H NMR ($CDCl_3$): 2.17 (quint, 2H, J= 6.7), 2.35 (t, 2H, J= 6.7), 2.68 (s, 3H), 4.49 (t, 2H, J= 6.7), 7.03 (d, 1H, J= 8.4), 7.17 (t, 1H, J= 8.4), 7.65 (d, 1H, J= 8.4), 7.85 (s, 1H). Picrate, m.p.: 199-201 °C (EtOH).

30.) 7-methyl-1-(2-propenyl) 1H-benzoimidazole

2.56 g of the reaction mixture containing the quaternary salt obtained according to point 16.) is reacted according to point 28.) and processed. Yield: 1.24 g (90 %). ^{1}H NMR ($CDCl_3$): 2.63 (s, 3H), 4.78-4.99 (m, 3H), 5.17-5.28 (*m, 1H), 5.96-6.17 (m, 1H), 7.00 (d, 1H, J= 7.3), 7.15 (t, 1H, J= 7.3), 7.65 (d, 1H, J= 7.3), 7.81 (s, 1H).

31.) 4-(2-propenyl)-4H-1,2,4-triazole oxalate

1.22 g 1H-1,2,4-triazole-1-propanoic-acid nitrile and 1.3 g allyl bromide in 5 ml acetonitrile are boiled under reflux cooling for 10 hours. The raw product obtained is dissolved in 10 ml acetone. It is treated in a solution prepared with 1.26 g (0.01 mol) oxalic-acid dihydrate and 3 ml EtOH, cooled, filtered, washed with acetone, dried. Yield: 1.39 g (73 %), m.p.: 97-99 %. Base: boiling point$_3$: 167-170 °C.

32.) 4-(phenylmethyl)-4H-1,2,4 triazole

3.40 g of the product of point 15.) and 1 g NaOH in a solution prepared with 30 ml methanol are boiled under reflux cooling for 0.5 hour. After recooling, the reaction mixture is stirred with 8 g silica gel for 0.5 hour at room temperature, dried, and evaporated. The raw product is taken up in 30 ml chloroform, stirred with 5 g silica gel and 2 g clarifying carbon for 0.5 hour and filtered, and the filtrate is evaporated. Yield: 1.46 g (92 %). Recrystallizing from an ether-hexane mixture: [m.p.:] 113-114 °C.

33.) 4H-1,2,4-triazole-4-butanoic-acid nitrile

1.69 g of the product of point 5.) and 1.48 g 4-bromobutyronitrile in 5 ml nitromethane are boiled for 22 hours under reflux cooling. It is evaporated, the quaternary salt is treated according to point 32.), the raw product is dissolved in 10 ml acetone, treated with a solution of 1.26 g (0.01 mol) oxalic-acid dihydrate in 3ml ethanol solution, and washed with acetone. Yield: 1.45 g (64 %), m.p.: 93-95 °C (acetone). Base: ^{1}H NMR ($CDCl_3$): 2.20 (quint, 2H), 2.43 (t, 2H), 4.26 (t, 2H), 8.23 (s, 2H).

34.) 1-methyl 1H-imidazole

68.08 g imidazole, 105 g ethyl acrylate, and 1.39 g TBD are stirred for 1 hour in 100 ml acetonitrile. 130 g dimethyl sulfate is dripped in over 0.5 hour. It is boiled for 1 hour under reflux cooling. The reaction mixture is evaporated. It is dissolved in 200 ml water and treated with a mixture of 100 g sodium hydroxide and

100 ml water. It is stirred for one hour at room temperature, then extracted 5 times with 100 ml ethyl acetate. The organic phase is dried and evaporated, and the residue is distilled. Yield: 72.2 g (88 %), b.p. 195-197 °C.

35.) 1-(phenylmethyl) 1H-benzoimidazole

11.8 g benzoimidazole, 11 g ethyl acrylate, and 0.14 g TBD are boiled in 30 ml acetonitrile under reflux cooling for 1 hour, then 13 g benzyl chloride is added to it, and it is boiled for 20 more hours. The solvent is distilled off, the evaporation residue is taken up in 50 ml water and treated with 10 g NaOH and 15 ml water. It is stirred for 1 hour at room temperature, then for 2 hours at 0-4 °C. The precipitate product is filtered, washed with water, and dried. Yield: 14.9 g (71 %), m.p.: 116-118 °C.

36.) 1-ethyl-5-phenyl 1H-imidazole

5 g 4-phenyl imidazole, 3.7 g ethyl acrylate, and 0.14 g TBD in 20 ml acetonitrile are stirred for 1 hour, then 5.6 g diethyl sulfate are added, and it is evaporated under reflux cooling for 20 hours, then evaporated. The evaporation residue is taken up in 50 ml water and treated with 3.2 g sodium-hydroxide at room temperature. After 1 hour of stirring, the reaction mixture is extracted twice with 30 ml ethyl acetate. The combined organic phase is dried and evaporated. Yield: 5.4 g (89 %) of product, b.p. 109-110 °C (0.4 mmHg).

37.) 5-phenyl-1-(2-propenyl) 1H-imidazole

In a manner similar to example 36.), starting from 2.88 g 4-phenyl imidazole, 2.2 g ethyl acrylate, 0.07 g TBD, and 3.6 g allyl bromide, 3.17 g (86 %) of product is obtained. ^{1}H NMR ($CDCl_3$): 4.52-4.61 (m, 2H), 4.97-5.30 (m, 2H), 5.83-60.5 (m, 1H), 7.11 (d, 1H, J= 1.1), 7.34-7.46 (m, 5H), 7.57 (d, 1H, J= 1.1). Picrate, m.p.: 127-128 °C (ethanol).

38.) 4-(2-propenyl)-4H-1,2,4-triazole oxalate

3.45 g 1,2,4-triazole, 4 ml acryl nitrile, and 0.14 g TBD in 10 ml acetonitrile are stirred for 3 hours, then 10 g allyl bromide is added to it over 4 hours under reflux cooling. It is boiled and evaporated. The evaporation residue is treated as described in example 36.). The raw product is dissolved in 50 ml acetone and treated with a boiling solution prepared from 6.3 g oxalic-acid dihydrate and 15 ml ethanol. It is cooled and stirred for 4 hours, filtered, and washed with acetonitrile. Yield: 6.33 g (64 %) of an isomer-pure crystalline product, m.p.: 97-99 °C.

39.) 4-butyl-4H-1,2,4-triazole oxalate

6.76 g raw 1H-1,2,4-triazole-1-propanoic-acid ethyl ester, 20 ml butyl bromide, and 0.30 g sodium iodide in 20 ml nitromethane are boiled for 20 hours under reflux cooling, then evaporated. The quaternary salt is converted according to point 38.). Yield: 4.98 g (58 %), m.p.: 109-11 °C. Base: ^{1}H NMR ($CDCl_3$): 0.95 (t, 3H), 1.36 (sext, 2H), 1.80 (quint, 2H), 4.02 (t, 2H), 8.16 (s, 2H).

40.) 4-(2-butyl) 4H-1,2,4-triazole

1.22 g raw 1H-1,2,4-triazole-1-propanoic-acid nitrile, 5.4 ml 2-bromobutane, and 0.15 g sodium iodide in 5 ml nitromethane are boiled for 60 hours under reflux cooling, then evaporated. The residue is purified according to point 36.) by column chromatography, eluting with a 9:1 (by volume) mixture of acetone and methanol. Yield: 0.58 g (47 %). ^{1}H NMR ($CDCl_3$): 0.83 (t, 3H), 1.48 (d, 3H), 1.77 (quint, 2H), 4.15 (sext, 1H), 8.13 (s, 2H).

41.) 1-(phenylmethyl)-1H-imidazole-5-carbonic-acid ethyl ester

1.26 g imidazole-4-carbonic-acid methyl ester, 1 g crotonic-acid nitrile, and 0.03 g TBD are boiled under reflux cooling for 1 hour, then 1.71 g benzyl bromide is added, and it is stirred for 60 more hours, the evaporated. The evaporation yield is taken up in 10 ml of a 2-M ethanol solution of Na ethylate, stirred for 10 minutes at room temperature, then for 20 minutes at 60 °C. Treated with 20 ml of a 10% ammonium-chloride solution. Stirred for 20 hours at room temperature, filtered, washed with cold water, and dried. Yield: 1.82 g (79%), m.p.: 64-65 °C.

42.) 1-methyl-1H-imidazole-5-carbonic-acid ethyl ester

1.26 g imidazole-4-carbonic-acid methyl ester, 1 g crotonic-acid nitrile, and 0.03 TBD are boiled for 1 hour under reflux cooling, then 1.05 ml (1.38 g) dimethyl sulfate is added, and it is boiled for 3 more hours and evaporated. The residue is taken up in 10 ml of a 2-M ethanol solution of ammonium chloride, stirred for 60 minutes at room temperature, cooled, treated with 20 ml of a 10% ammonium-chloride solution, stirred for 20 hours, filtered, and washed. Yield: 1.05 g (75 %), m.p.: 54-56 °C.

43.) 4-nitro-1-(3-oxobutyl) 1H-imidazole

2.26 g 4-nitroimidazole, 2 ml (1.71 g) methylvinyl ketone, and 0.14 g TBD are boiled in 25 ml acetonitrile under reflux cooling for 1 hour, then evaporated. The evaporation residue is treated with 10 ml of a 10% solution of ammonium chloride, cooled, filtered, and washed with water. Yield: 3.41 g (93 %), m.p.: 73-74.5 °C (EtOAc). ^{1}H NMR ($CDCl_3$): 2.10 (s, 3H), 3.11 (t, 2H), 4.22 (t, 2H), 7.83 (d, 1H), 8.36 (d, 1H).

44.) 7H-theophillin-7-propanoic-acid nitrile

3.6 g theophillin and 0.14 g TBD in 15 ml acrylonitrile for 120 hours and evaporated, and the raw product is treated according to point 43.). Yield: 4.3 g (95 %), m.p.: 159-161 °C. Recrystallizing from EtOAc: m.p.: 160-161 °C.

45.) 3-phenyl-1H-1,2,4-triazole-1-propanoic-acid nitrile

[Proceeding] according to point 44.) from 2.90 g 3-phenyl-1H-1,2,4-triazole. Yield: 3.65 g (92 %), m.p.: 86.5-88 °C. ^{1}H NMR ($CDCl_3$): 3.04 (t, 2H), 4.45 (t, 3H), 7.37-7.52 (m, 3H), 8.02-8.15 (m, 2H), 8.20 (s, 1H).

46.) 3-phenyl-1H-pyrazole-1-propanoic-acid nitrile

[Proceeding] according to point 44.) from 2.88 g 3-phenyl pyrazole. Yield: 3.43 g (87 %), m.p.: 51-53 °C (ether-hexane). ^{1}H NMR ($CDCl^3$): 3.00 (t, 2H), 4.42 (t, 2H), 6.58 (d, 1H), 7.30-7.48 (m, 3H), 7.52 (d, 1H), 7.78 m, 2H).

47.) 1H-benzotriazole-1-propanoic-acid nitrile and 2H-benzotriazole-2-propanoic-acid nitrile

Proceeding according to point 44.) from 2.38 g benzotriazole and purifying the raw product by chromatography (eluent: 95:5 chloroform:acetone). Yield: 2.26 g (66 %), m.p.: 78-80 °C.

48.) 1-methyl-5-nitro-1H-imidazole

a) 2.26 g 4-nitroimidazole, 1.6 ml acrylonitrile, and 0.07 g TBD in 10 ml acetonitrile are boiled for 8 hours under reflux cooling. 2.1 ml (2.78 g) dimethyl sulfate is added, and it is boiled for 3 more hours. The mixture is cooled, 3.16 ml (3.37 g) 7-methyl-1,5,7-triazabicyclo-[4,4,0]-dec-5-ene (7-Me-TBD) is stirred in over 0.5 hour at room temperature. It is evaporated on a column filled with silica gel and eluted with a mixture of ethyl acetate and acetone (2:1). Yield: 1.45 g (57 %), m.p.: 52-54 °C.

b) 1.45 g 4-nitro-1-(3-oxobutyl) 1H-imidazole and 0.76 ml (1.01 g) dimethyl sulfate in 5 ml acetonitrile are boiled for 4 hours under reflux cooling. It is cooled, and 2.76 g ground potassium carbonated is assed with mixing. It is stirred for 10 hours at room temperature, filtered, evaporated, and chromatographed according to what is described at point a). Yield: 0.87 g (87 %), m.p.: 53-55 °C.

49.) 7-(3-oxobutyl) 7H-theophillin

5.4 g theophillin, 2.7 ml (2.31 g) methylvinyl ketone, and 0.14 g TBD in 20 ml acetonitrile are boiled for 3 hours under reflux cooling. It is evaporated, and the residue is taken up in 40 ml of a 10% HCl solution and dried at 60-70 °C. The filtrate is cooled, the germicidal effect is set to pH 8 with a 25% ammonium solution. It is crystallized at 0-4 °C, filtered, and washed. Yield: 6.31 g (84 %), m.p.: 138.5-140 °C (ethyl acetate).

50.) 5-methyl-5H-imidazo-(4,5-c)-pyridine oxalate

1.19 g 5-azabenzoimidazole, 1 ml (0.8 g) acrylonitrile, and 0.014 g TBD in 5 ml acetonitrile are stirred at room temperature for 0.5 hour, then 0.75 ml (1.7 g) methyl iodide is added, and it is stirred for 4 more hours. It is evaporated, 5 ml of a 20% sodium-hydroxide solution is added, and it is stirred for 4 more hours. A saturated salt solution is added, and it is extracted with 5-30 ml dichloromethane. The organic phase is dried, clarified, and evaporated, and the residue is dissolved in 10 ml acetone. It is treated with a solution prepared from 1.26 g oxalic acid in 5 ml boiling ethanol, cooled, filtered, and washed with acetone. Yield: 0.98 g (44 %), m.p.: 188-190 °C (EtOH).

51.) 5-(phenylmethyl)-5H-imidazo-(4,5-c) oxalate

Using 1.71 g benzyl bromide as the alkylizing agent according to point 50.) and a quaternarization time of 20 hours. Yield: 1.08 g (33 %), m.p.: 142-143.5 °C (EtOH).

52.) β-methyl-1H-imidazole-1-propanoic-acid nitrile

13.6 g imidazole, 18.5 ml (15 g) methacrylonitrile, and 0.3 g TBD in 30 ml acetonitrile are boiled under reflux cooling for 100 hours. 5-5 ml methacrylonitrile is added every 24 hours

to the mixture. It is evaporated. Yield: 27.96 g. ^{1}H NMR ($CDCl_3$): 1.29 (d, 3H), 3.08 (sext, 1H), 4.13 (d, 2H), 7.05 (s, 1H), 7.56 (s, 1H).

53.) 1-(2-phthalimidoethyl) 1H-imidazole

1.36 g imidazole, 1.7 ml (1.32 g) acrylonitrile, and 0.03 g TBD in acetonitrile are boiled under reflux cooling for 0.5 hour. 5.1 g 2-bromoethyl phthalimide is added, and it is boiled for 8 more hours and cooled. 35 ml ether is added. It is crystallized at 0-5 °C and filtered. The raw quaternary salt is dissolved in 200 ml methanol. 2.88 ml (3.06 g) 7-Me-TBD is added to it, and it is stirred for 5 hours, evaporated, treated with 20 ml of a 100-g/l solution of ammonium chloride, stirred at 0-5 °C, filtered, washed, and dried. Yield: 4.39 g (91 %), m.p.: 165-167 °C (2-propanol).

54.) 5-phenyl-1-methyl 1H-pyrazole

1.97 g 3-phenyl-1H-pyrazole-1-propanoic-acid nitrile and 1.05 ml (1.38 g) dimethyl sulfate in 5 ml acetonitrile are boiled for 15 hours under reflux cooling. 1.58 ml (1.69 g) 7-Me-TBD are added at 10-15 °C, it is stirred for 5 hours and evaporated, and the residue is treated with 10 ml of a 100 g/l solution of ammonium chloride. It is extracted 3 times with 20 ml chloroform. The combined organic phase is dried, clarified, and evaporated, and the residue (1.37 g) is purified by distillation. Yield: 0.94 g (59 %), $b.p._{12}$ 118 °C.

55.) 3-phenyl-4-methyl 4H-1,2,4-triazole

1.98 g 3-phenyl-1H-1,2,4-triazole-1-propanoic-acid nitrile is treated according to example 54.), and the raw product is crystallized from petroleum ether / ethyl acetate.
Yield: 1.00 g (63 %), m.p.: 111-113 °C.

56.) 1,3,9-trimethyl xanthine (isocaffeine)

1.4 g 7H-theophillin-7-propanoic-acid nitrile and 0.7 ml (0.88 g) dimethyl sulfate in 5 ml acetonitrile are boiled for 15 hours under reflux cooling. 1 ml (1.07 g) 7-Me-TBD is added at 10-15 °C, it is stirred at room temperature and evaporated, and 5 ml ethanol is added, it is stirred at 0-5 °C, filtered, washed with a mixture of cold ethanol and water. Yield: 0.91 g (78 %), m.p.: 294-295 °C (EtOH-water).

57.) 4-acetamino-1-(2-cyanoethyl)-3-(phenylmethyl)-imidazolium bromide

1.78 g (0.01 mol) 4-acetamino-1H-imidazole-1-propanoic-acid nitrile and 2.56 g (0.015 mol) benzyl bromide in 20 ml Me CN are boiled for 16 hours under reflux cooling, then cooled, and the precipitated crystalline product is filtered, washed with acetone, and dried. Yield: 3.05 g (87 %), m.p.: 194-196 °C.

58.) 3-(N-acetyl-N-(1-phenylmethyl-1H-imidazole-5-yl))amino-propanoic-acid nitrile and 5-acetamino-1-(phenylmethyl) 1H-imidazole

2.4 g (6.9 mmol) 4-acetamino-1-(2-cyanoethyl)-3-(phenylmethyl)-imidazolium bromide and 2.1 ml (2.13 g, 14 mmol) diazabicyclo-undecene (DBU) in 10 ml MeCN are stirred at 50 °C for 0.5 hour. It is evaporated, the evaporation residue is treated with

15 ml of a 10% ammonium-chloride solution, and it is extracted 3 times with 20 ml dichloromethane. The organic phase is evaporated, the residue is eluted in a silica-gel column with 9:1 acetone:methanol. The first fraction obtained is 3-(N-acetyl-N-(1-(phenylmethyl)-1H-imidazole-5-yl))amino-propanoic-acid nitrile): 0.72 g, m.p.: 112-114 °C (EtOAc). ^{1}H NMR (DMSO-d_6): 1.36 (s, 3H), 2.56-2.72 (m, 2H), 2.73-2.91 (m, 1H), 4.02-4.22 (m, 1H), 5.08 (m, 2H), 6.94 (s, 1H), 7.19-7.43 (m, 5H), 7.92 (s, 1H). MS (EI$^+$, 70 eV) (m/z, %): 268 (M^+, 3), 226 (8), 186 (27), 91 (100). IR (KBr): 1574, 1671, 2255 cm^{-1}. The second fraction is 5-acetamino-1-(phenylmethyl)-1H-imidazole: 0.40 g, m.p.: 149-151 °C (10:1 toluene:n-butanol). ^{1}H NMR (DMSO-d_6): 1.98 (s, 3H), 5.05 (s, 2H), 6.75 (s, 1H), 7.08-7.20 (m, 2H), 7.26-7.41 (m, 3H), 7.49 (s, 1H), 9.59 (NH). MS (EI$^+$, 70 eV) (m/z, %): 215 (M^+, 9), 173 (18), 91 (100).

59.) 5-acetamino-1-(phenylmethyl) 1H-imidazole

2.96 g (8.5 mmol) 4-acetamino-1-(2-cyanoethyl)-3-(phenylmethyl)-imidazolium bromide is added to 50 ml of a 2-M methanol solution of MeONa (0.1 mol MeONa) with stirring. After 5 minutes, 4.9 g ammonium chloride is added to the mixture, then 10 g silica gel is added, and it is mixed for 0.5 hour. It is filtered, the filtrate is evaporated, and the residue is crystallized from a toluene:n-butanol mixture. Yield: 1.20 g (66 %), m.p.: 150-152 °C.

60.) E-1-(2-cyanoethyl)-1H-imidazole-4-propenic-acid ethyl ester

3.32 g (0.02 mol) E-1H-imidazole-4-propenic-acid ethyl ester (urocanic-acid ethyl ester), 1.45 ml (1.17 g, 0.022 mol) acrylonitrile, and 0.14 g (1 mmol) TBD in MeCN are stirred at room temperature for 10 hours, then crystallized at -18 °C for 24 hours and filtered. Yield: 4.03 g (92 %), m.p.: 120-121.5 °C (EtOH). ^{1}H NMR ($CDCl_3$): 1.31 (t, 3H), 2.84 (t, 2H), 4.16-4.33 (m, 4H), 6.57 (d, 1H, J= 15 Hz), 7.22 (m, 1H), 7.55 (d, 1H, J= 15 Hz), 7.59 (m, 1H). MS (EI$^+$, 70 eV) (m/z, %): 219 (M^+, 27), 174 (100), 147 (46), 104 (25).

61.) 4-nitro-1H-benzoimidazole-1-propanoic-acid nitrile

3.1 g (19 mmol) 4-nitro-benzoimidazole, 20 ml acrylonitrile, 2.45 g (20 mmol) 4-(dimethylamino)-pyridine, and 0.14 g (1 mmol) TBD in 10 ml MeCN are stirred at 70 °C for 6 hours, then cooled, crystallized at -18 °C, and filtered. Yield: 3.8 g (88 %), m.p.: 195-196 °C (acetone). ^{1}H NMR (DMSO-d_6): 3.19 (t, 2H), 4.72 (t, 2H), 7.52 (t, 1H), 8.10 (dd, 1H), 8.25 (dd, 1H), 8.63 (s, 1H). MS (EI$^+$. 70 eV) (m/z, %): 216 (M^+, 100), 186 (68), 146 (33), 118 (94).

62.) α-4-dimethyl-1H-imidazole-1-propanoic-acid-nitrile oxalate

1.64 g (0.02 mol) 4-methyl imidazole and 1.45 ml (1.17 g, 0.022 mol) acrylonitrile) in 8 ml MeCN are quaternized at a temperature of 80 °C for 5 hours, then evaporated, and the evaporation residue is dissolved in 5 ml acetone and treated with a solution of 2.52 g (0.02 mol) oxalic-acid dihydrate and 5 ml EtOH. It is cooled, and the precipitated crystals are filtered and washed with acetone. Yield: 3.11 g (65 %), m.p.: 104-105.5 °C (EtOH). ^{1}H NMR (DMSO-d_6): 1.51 (d, 3H, J- 6.8 Hz), 2.20 (d, 3H, J= 0.9 Hz), 3.17 (d, 2H, J= 6.6 Hz), 4.77 (sext, 1H, J= 6.7 Hz), 7.38 (m, 1H), 8.51 (d, 1H, J=1.5 Hz). MS (EI$^+$, 70 eV) (m/z, %): 149 (base M^+, 48), 109 (100), 81 (43).

63.) E-1-(2-propenyl)-1H-imidazole-5-propenic-acid ethyl ester

3.29 g (0.015 mol) E-1-(2-cyanoethyl)-1H-imidazole-4-propenic-acid ethyl ester, 1.7 ml (2.43 g, 0.02 mol) allyl bromide, and 0.5 g NaI in 20 ml MeCN are quaternized at a temperature of 50 °C for 120 hours. After cooling, 3 ml (3.04 g, 0.02 mol) diazabicyclo-

undecene (DBU) is added at a temperature of 10 °C, then stirred at a temperature of 25 °C for 0.5 hour and evaporated. The evaporation residue is treated with 30 ml of a 10% ammonium-chloride solution, then extracted 3 times with 20 ml dichloromethane. The organic phase is evaporated, and the evaporation residue is eluted with acetone in a short silica-gel column. Yield: 2.75 g (89 %), m.p.: 120 °C.

64.) 1-(pivaloyloxy)methyl-5-methyl 1H-imidazole

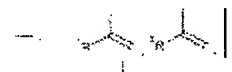
2.39 g (0.01 mol) α-4-dimethyl-1H-imidazole-1-propanoic-acid-nitrile oxalate is added to 35 ml of a 10% ammonia solution at a temperature of 10 °C, with stirring, then the mixture obtained is extracted 3 times with 20 ml dichloromethane. The organic phase is evaporated, 5 ml MeCN, 1.6 ml (1.66 g, 0.011 mol) pivalinic-acid chloromethyl ester, and 0.2 g NaI are added to the evaporation residue, it is stirred at a temperature of 25 °C for 10 days, then diluted with 15 ml EtOC, cooled, and filtered. The 2.4 g of imidazolium salt obtained are dissolved in 20 ml MeCN, and it is treated at a temperature of 10 °C with 1.65 ml (1.67 g, 0.011 mol) diazabicyclo-undecene (DBU), then stirred at a temperature of 25 °C for 0.5 hour. It is evaporated, and the residue is treated with 30 ml of a 10% ammonium-chloride solution, then extracted 3 times with 20 ml dichloromethane. The organic phase is evaporated, and the residue is chromatographed in a silica-gel column with a mixture of EtOAc and MeOH. Yield: 1.02 g (52 %), colorless oil. ^{1}H NMR ($CDCl_3$): 1.18 (s, 9H), 2.27 (d, 3H, J= 1.0 Hz), 5.80 (s, 2H), 6.78 (m, 1H), 7.61 (m, 1H). MS (EI$^+$, 70 eV) (m/z, %): 196 (M^+, 7), 95 (20), 94 (28), 57 (100).

65.) 5-bromo-1-methyl 1H-imidazole

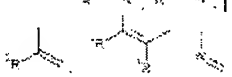
2.94 g (0.02 mol) 4-bromoimidazole, 1.79 ml (1.48 g, 0.022 mol) crotonic-acid nitrile, and 0.14 g (1 mmol) TBD in 15 ml acetonitrile are stirred at 80 °C for 3 hours, 2.1 ml (2.77 g, 0.022 mol) dimethyl sulfate is added, then it is stirred at this temperature for 3 more hours. It is cooled to 10 °C, 3.3 ml (3.34 g, 0.022 mol) DBU, is stirred for half an hour at 25 °C, and the mixture is evaporated. The residue is treated with 30 ml of a 10% aqueous solution of ammonium-chloride solution and extracted 3 times with 20 ml dichloromethane. The organic phase is evaporated, and the residue is eluted on silica gel with acetone. Yield: 2.74 g (85%). Melting point: 44-46 °C.

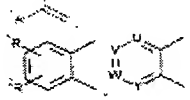
Claims

1. A process for preparing N-alkylized azoles containing at least two nitrogen atoms (general formula 1), where

the meaning of A is 

the meaning of B is 

the meaning of D is 

the meaning of BD is 

the meaning of R^1, R^2, and R^3 is H; a C_{1-4} alkyl, possibly substituted;

(substituted phenyl); $NHCOC_{1-4}$ alkyl; $COOC_{1-4}$ alkyl

the meaning of U, V, W, Y, and Z is CH; N; CO; CS, $N–C_{1-8}$alkyl; $C–OC_{1-4}$ alkyl; $C\text{-}OC_{1-4}$ alkyl; $C–N(C_{1-4}$ alkyl$)_2$

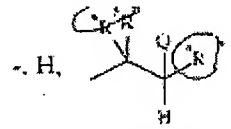
the meaning of n is 0, 1

the meaning of X is a chlorine, bromine, or iodine atom; C_{1-4} alkyl–SO_3; OSO_3R^7 C_{1-4} fluoridated alkyl–SO_3, (substituted) phenyl–SO_3

the meaning of R^7 is –; possibly substituted C_{1-8} alkyl; N-containing heteroaryl

the meaning of R^8 is

illegible



the meaning of R^4, R^5, and R^6 is H; alkyl; cycloalkyl; Q

the meaning of Q is CN; $COOC_{1-4}$ alkyl; COC_{1-4} alkyl; CO (substituted) phenyl; SO_2C_{1-4} alkyl; SO_2 (substituted) phenyl

azoles containing at least two unsubstituted nitrogen atoms (general formula 2, where the meaning of A, B, and D is as above) and olefins with an electron-absorbing group (general formula 3, meaning of R^4, R^5, and R^6 as above, in a reaction, characterized in that a.) the reaction is run is the presence of substituted amidine (general formula 4, where the meaning of E, J, and L is –, H, an aliphatic ring reside, an N-containing aliphatic ring residue) functioning as a base and or a transfer reagent.

b.) the N-monoalkyl azole obtained (general formula 5, where the meaning of A, B, D, R^4, R^5, and R^6 is as above), some alkylizing agent (general formula 6, where the meaning of X and R^7 is as above), possibly in the presence of an alkali-halide catalyst, the quaternary azolium salt (general formula 7, where the meaning of A, B, D, Q, X, R^4, R^5, R^6, and R^7 is as above, and the (substituted) ethyl substituent containing the electron-absorbing group Q is split off selectively with an appropriately selected base.

2. A process according to claim 1/a above, characterized in that 1,5,7-triazabicyclo-[4,4,0]-dec-5-ene or 7-methyl-1,5,7-triazabicyclo-[4,4,0]-dec-5-ene is used appropriately as a basic catalyst, by itself or applied to a polymer carrier.

3. A process according to claims 1/a and 2 above, characterized in that the ethylene derivative is appropriately used in a molar excess, possibly as a solvent.

4. A process according to claims 1/a, 2, and 3 above, characterized in that the product is isolated by evaporating the mixture, treating it with water, a solution of an inorganic aqueous salt, appropriately ammonium chloride or ammonium carbonate, and filtering.

5. A process according to claim 1/b, characterized in that the Michael adduct (general formula 5) is made to react with 0.9-10, appropriately 1-5 equivalents of alkylizing agent, possibly without isolation, *in situ*.

6. A process according to either of claims 1/b and 5, characterized in that the azolium salt formed (general formula 7) is isolated by evaporating the reaction mixture or diluting it with an aprotic solvent and filtration.

7. A process according to any of claims 1/b, 5, and 6, characterized in that the azolium salt is treated without isolation or by isolation in an alcohol and/or aqueous solution at 0-100 °C, with 0.95-5 equivalents of a base.

8. The process according to claims 1/b and 5-7, characterized in that the product is isolated by evaporation, treating it with water, an aqueous solution of an inorganic salt, appropriately ammonium chloride or ammonium carbonate, and filtering.

9. A process according to claims 1/b and 5-8, characterized in that the product is isolated by evaporating the mixture, stirring the residue with water, and extracting it with a water-immiscible organic solvent.

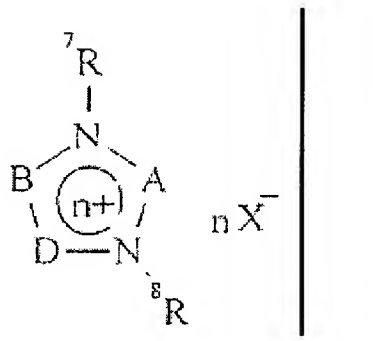
10. A process according to claims 1-9, characterized in that the Michael addition and/or alkylization is performed using a polar aprotic solvent, appropriately acetonitrile or nitromethane at 0-150 °C, appropriately 20-120 °C.

[2 signatures]

+ 1 page of drawings
[signature]

Diagrams

ANNOUNCEMENT COPY



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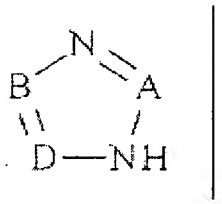

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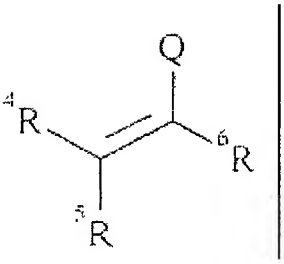

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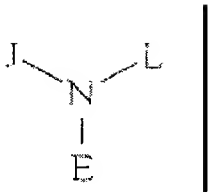

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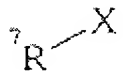

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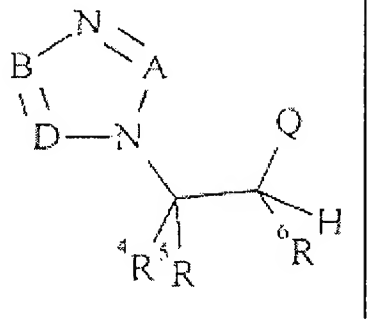

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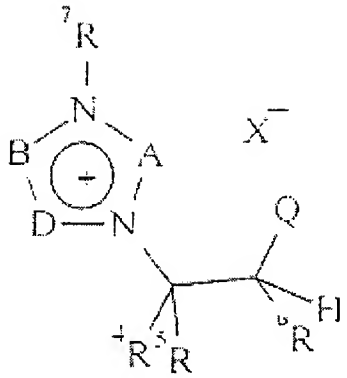

7.

[2 signatures]

Lapsed

HPO e-register (in Hungarian)

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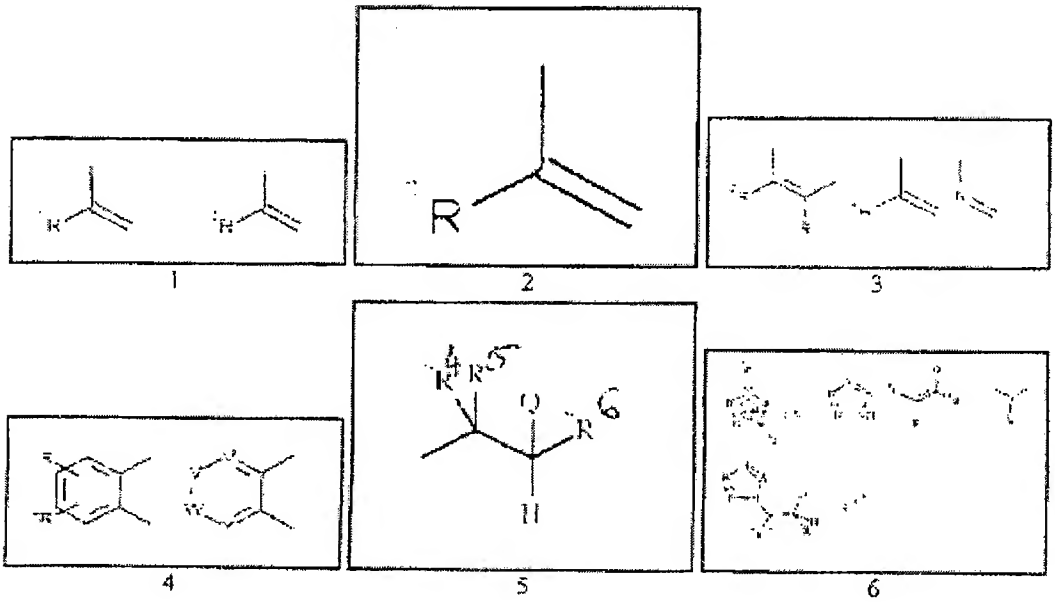
IPC: C07D-233/10; C07D-233/14; C07D-233/16; C07D-233/18

Hungarian title: **Eljárás szubsztituált nitrogéntartalmú, heterociklusos vegyületek szintézisére**

English title: PROCESS FOR THE PREPARATION OF SUBSTITUTED, NITROGEN CONTAINING HETEROCYCLIC COMPOUNDS

Applicant and inventor: Horváth András, Tiszadob (HU), 80%
Salamon Zoltán, Debrecen (HU), 20%

Representative: Salamon Zoltán, Debrecen (HU)



Abstract (first publication):

The object of the invention is a process for producing azoles of general formula (1) in the diagram

The meaning of A is

The meaning of B is

The meaning of D is

The meaning of BD is

The meaning of R^1, R^2, and R^3 is H, possibly a substituted C_{1-4} alkyl, (substitute) phenyl, $NHCOC_{1-4}$alkyl, or $COOC_{1-4}$alkyl; the meaning of U, V, W, Y, and Z is CH, N, CO, CS, NC_{1-8}alkyl, COC_{1-4}alkyl, CSC_{1-4}alkyl, or $CN(C_{1-4}alkyl)_2$; the meaning of n is 0 or 1; the meaning of X is a chlorine, bromine, or iodine atom, C_{1-4}alkyl SO_3, OSO_3R^7, C_{1-4} fluoridated alkyl SO_3, or (substituted) phenyl SO_3; the meaning of R^7 is –, H, possibly a substituted C_{1-8} alkyl or N-containing heteroaryl; the meaning of R^8 is – or H; the meaning of R^4, R^5, or R^6 is H, alkyl, cycloalkyl, or Q; the meaning of Q is CN, $COOC_{1-4}$alkyl, COC_{1-4}alkyl, CO (substituted) phenyl, SO_2C_{1-4}alkyl or SO_2 (substituted) phenyl.

According to the procedure, the unsubstituted azole (general formula 2) containing at least two N atoms

is made to react in the presence of a catalyst of the organic-amidine type (general formula 4, where the meaning of E, J, and L, is H, an aliphatic ring residue, or an N-containing aliphatic ring residue) in a polar, aprotic solvent or solvents, with substituted ethylene derivatives with an electron-absorbing group Q (general formula 3), while proceeding according to the process, the product (general formula 1 ($n = 0$, $R^8 = -$), is obtained by proceeding according to process B), making the N-monosubstituted azole (general formula 5) in a polar solvent by adding a catalyst with a halogen atom, and an alkylizing agent (general formula 6), the a base).

Measures

0. Data publication (A0)
Measure Date: 1995.04.04 *Announcement:* 1995.05.29 (AA1A Communication of patent application data)

5. Publication of patent application (CV)
Measure Date: 1999.03.30 *Announcement:* 1999.05.28 (BB9A Publication of patent applications)

8. Lapse of provisional patent protection due to non-payment of fees (EF)
Measure Date: 2000.01.13 *Reception:* 2000.01.19 *Announcement:* 2000.02.28 (FD9A Lapse of provisional patent protection due to non-payment of fees)

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UNION OF SOVIET SOCIALIST REPUBLICS
USSR STATE COMMITTEE FOR INVENTIONS AND DISCOVERIES

(19) **SU** (11) **851,940 A1**

(51) 4 C 07 D 471/04; A 61 K 31/395

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DESCRIPTION OF AN INVENTION
FOR AN AUTHORSHIP CERTIFICATE

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(22) March 20, 1980

(46) April 30, 1988, Bulletin No. 16

(71) Institute of Physicoorganic Chemistry and Coal Chemistry of the Ukrainian SSR Academy of Sciences, and the Zaporozh'ye Medical Institute

(72) O. G. Eilazyan, K. M. Khabarov, Yu. M. Yutilov, and P. N. Steblyuk

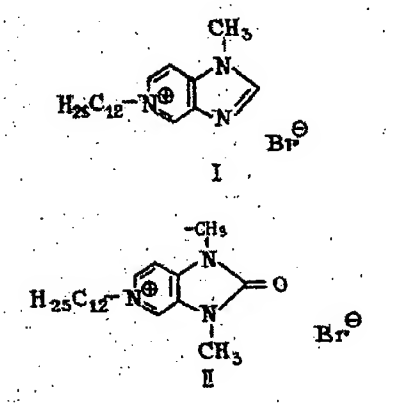
(53) 547.836.3 (088.8)

(56) U.S. Patent No. 3,919,193, classification 260-211.5, 1975.

M. D. Mashkovskii, *Medicinal Drugs*, Meditsina Publishers, Moscow, 1972, Vol. 2, p. 340.

(54) QUATERNARY SALTS OF IMIDAZO[4,5-*c*]PYRIDINIUM EXHIBITING ANTIMICROBIAL AND FUNGISTATIC ACTIVITY

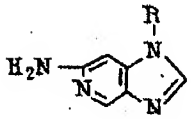
(57) Quaternary salts of imidazo[4,5-*c*]pyridinium having formula I or II:



which exhibit antimicrobial and fungistatic activity.

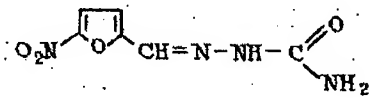
The invention relates to new biologically active chemical compounds, specifically to quaternary salts of imidazo[4,5-*c*]-pyridinium, which exhibit antimicrobial and fungistatic activity.

Derivatives of 4-oxy-7-amino-imidazo[4,5-*c*]pyridine having the following formula are known:



where R is β-ribofuranosyl or the 2′,3′,5′–O–C_1 to C_8 acylated analogue, which exhibit antiviral activity.

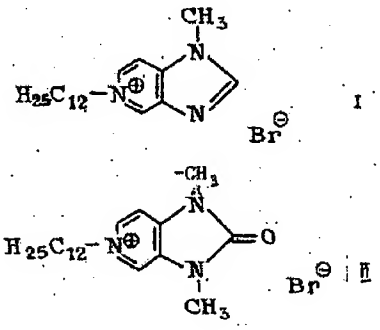
Also known is the drug furacilin:



which is used as an antimicrobial agent.

The object of the invention is to expand the toolkit of agents for acting on the living organism.

The stated object is attained by the novel chemical compounds — quaternary salts of imidazo[4,5-*c*]pyridinium having formula I or II:



which are obtained by reacting dodecylbromide with the corresponding imidazo[4,5-*c*]pyridine while it is being heated in a solvent medium.

The end product obtained — quaternary salts of imidazo[4,5-*c*]pyridinium — comprises colorless crystalline substances that dissolve well in water and alcohol, the solutions of which have surfactant (detergent) properties.

Example 1.

5-Lauryl-1-methylimidazo[4,5-*c*]pyridinium bromide (I), EYu-196.

A quantity of 10 mmol of 1-methylimidazo[4,5-*c*]pyridine is dissolved in 15 mL of absolute benzene, 12.5 mmol of dodecylbromide is added, and [the resulting solution] is boiled for 2.5 hr on an oil bath at a temperature of 110°C. After cooling, the precipitate is filtered off and recrystallized from nitromethane. Yield is 82%, and m.p. is 63–64°C (nitromethane).

Found: 59.48%, H 8.51%, N 10.9%, Br 20.7%.

$C_{19}H_{32}BrN_3$

Calculated: C 59.67%, H 8.43%, N 10.98%, Br 20.89%.

UV spectrum: λ_{max}, nm (log ε) 216 (422), 240 (3.20), 266 (3.42).

Example 2.

4-Amino-5-lauryl-1,3-dimethylimidazo[4,5-*c*]pyridinium-2-one bromide (II), KhYu-2.

A solution of 1 mmol of 4-amino-1,3-dimethylimidazo[4,5-*c*]pyridin-2-one in 0.5 mL of sulfolane and 1.2–1.25 mmol dodecylbromide is heated at 160–170°C on an oil bath for 1 hr, the reaction mass is cooled, and the precipitate is filtered off, washed with benzene and ether, and dried. Yield is 0.32 g (73%). M.p. is 134–135°C (alcohol with ether).

Found: C 55.7%, H 7.9%, Br 19.1%.

$C_{20}H_{35}BrN$.

Calculated: C 56.2%, H 8.2%, Br 18.7%.

Infrared spectrum: 3380 cm^{-1} (NH), 1730 cm^{-1} (CO).

UV spectrum: λ_{max}, nm (log ε) 222 (455):260(3.85), 292 (3.62).

The activity of quaternary salts of imidazo[4,5-*c*]pyridinium EYu-196 and KhYu-2 on the antimicrobial and fungistatic activity of compounds was studied by the method of doubling serial dilutions on a liquid medium over a range that included up to five strains of microorganisms. Hottinger broth (*p*H 7.2–7.4) was used to culture the bacteria. The microbial load for the bacteria was 2.5×10^5 cells of an 18-hr agar culture in 1 mL of medium. The highest concentration tested was 200 μg/mL.

Sabouraud's medium (*p*H 6.0–6.8) was used to grow the fungi. The load was 500,000 reproductive bodies per milliliter. The highest tested concentration was 200 μg/mL.

The antimicrobial activity of the compounds was judged by the minimum bacteriostatic and fungistatic concentration of the chemical compounds, expressed in μg/mL.

Furacilin was taken as the reference.

As one sees from the data, the drugs tested have more potent action toward staphylococcus by a factor of 2 (EYu-196), toward anthracoid by factors of 16 and 4 (EYu-196 and KhYu-2, respectively), toward colon bacillus by factors of 16 and 2, and toward *Candida abb* by factors of 16 and 4. With regard to *Bacillus pyocyaneus*, both drugs act at the standard level.

Test Results for Antimicrobial and Fungistatic Activity
(the Minimum Bacteriostatic Concentration
Is Specified in μg/mL)

No.	Strains of microorganisms and fungi	Designators of tested compounds		
		EYu-196	KhYu-2	Furacilin
1	*Staphylococcus aureus* 209p	2	8	4
2	*Bacillus anthracoides* 1312	2	8	31
3	*Escherichia coli* 675	1	8	16
4	*Pseudomonas aureginosa* 165	250	250	250
5	*Candida albicans*	4	16	63

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(51)4 C 07 D 471/04; A 01 N 43/50

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DESCRIPTION OF AN INVENTION
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(71) Institute of Physicoorganic Chemistry and Coal Chemistry of the Ukrainian SSR Academy of Sciences, and All-Union Scientific Research Institute of Plant-Protection Chemicals

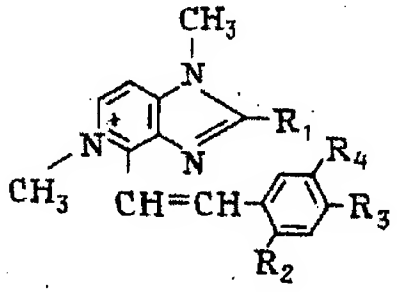
(72) Yu. M. Yutilov, A. G. Ignatenko, L. Ye. Mikhailova, Ye. I. Andreyeva, and G. V. Bobkova

(53) 547.859 (088.8)

(56) U.S. Patent No. 3,759,933, classification C 07 d 31/40, published 1973.

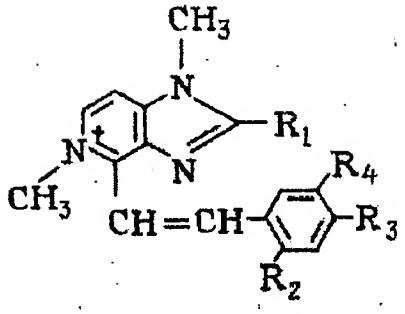
(54) STYRYL DERIVATIVES OF IMIDAZO[4,5-*c*]PYRIDINIUM EXHIBITING FUNGICIDAL ACTIVITY

(57) Styryl derivatives of imidazo[4,5-*c*]pyridinium iodide having the general formula:



where a) R_1 is CH_3, R_2 and R_3 are OCH_3, and R^3 is H;
b) R_1 is CH_3, R_2 and R_3 are H, and R_3 is $N(CH_3)_2$;
c) R_1 is CH_3, R_2 and R_4 are H, and R_3 is OCH_3;
d) R_1 is Ph, R_2 and R_4 is OCH_3, and R_3 is H; and
e) R_1 is Ph, R_2 and R_4 are H, and R is $N(CH_3)_2$,
which exhibit fungicidal activity.

The invention relates to new chemical compounds, to styryl derivatives of imidazo[4,5-*c*]-pyridinium iodide having the general formula:



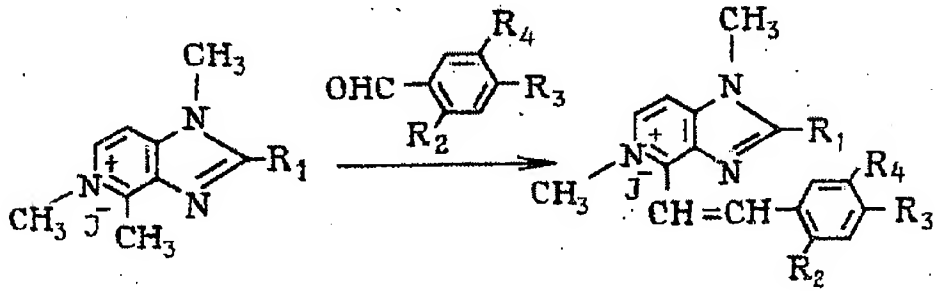
where a) R_1 is CH_3, R_2 and R_3 are OCH_3, and R_3 is H;
b) R_1 is CH_3, R_2 and R_3 are H, and R_3 is $N(CH_3)_2$;
c) R_1 is CH_3, R_2 and R_4 is H, and R_3 is OCH_3;
d) R_1 is Ph, R_2 and R_4 is OCH_3, and R_3 is H; and
e) R_1 is Ph, R_2 and R_4 are H, and R is $N(CH_3)_2$,
which have fungicidal activity.

This property makes it possible to propose the possibility that they can be used in agriculture.

Derivatives of imidazo[4,5-*c*]-pyridin-2-one exhibiting anti-inflammatory activity are known.

The object of the invention is to expand the range of fungicides.

This object is attained by new styryl imidazo[4,5-*c*]-pyridinium iodides having the cited general formula, which are obtained by reacting 4-methyl derivatives of imidazo[4,5-*c*]-pyridinium iodides with aromatic aldehydes in the presence of piperidine as a catalyst, by the following scheme:



where R_1–R_4 have the indicated meanings.

The new compounds obtained are colored solids that are soluble in water, alcohol, and acetone. The structure is confirmed by data of ultimate analysis.

Example 1. 1,2,5-Trimethyl-4-(*n'*-N',N'-dimethylaminostyryl)-imidazo-[4,5-*c*]-pyridinium iodide (IYu-6).

A quantity of 1.05 g (3.5×10^{-3} mol) of 1,2,4,5-tetramethylimidazo[4,5-*c*]pyridinium iodide and 0.7 g (4.5 mmol) of *n*-(dimethylamino)benzaldehyde are dissolved, while being heated, in 30 mL of *n*-butanol, 2 mL (2 mmol) of piperidine is added, and [the resulting mixture] is boiled on an oil bath at a temperature of 135–145°C for 2 hr. After cooling, the brick-red precipitate is filtered off and washed with ether; yield is 1.45 g (96.7%), and m.p. is 228–230°C (*n*-butanol).

Found: C 52.5%, H 5.4%, N 12.7%.

$C_{19}H_{23}N_4$.

Calculated: C 52.5%, H 5.3%, N 12.9%.

Example 2. 1,2,5-Trimethyl-4-(2,5-dimethoxystyryl)imidazo[4,5-*c*]pyridinium iodide (IYu-5).

[This compound] is obtained by analogy with Example 1, by proceeding from 1.05 g (3.5 mmol) of 1,2,4,5-tetramethylimidazo[4,5-*c*]pyridinium iodide and 0.7 [g] (4.2 mmol) of 2,5-dimethoxybenzaldehyde; yield is 1.5 g (96%), and m.p. is 184–185°C (*n*-butanol).

Found: C 50.5%, H 5.5%, N 9.2%.

$C_{19}H_{22}N_3O_2I$.

Calculated: C 50.5%, H 5.6%, N 9.3%.

Example 3. 1,2,5-Trimethyl-4-(*n'*-methoxystyryl)imidazo[4,5-*c*]pyridinium iodide (IYu-452).

[This compound] is obtained by analogy with Example 1, by proceeding from 1.05 g (3.5 mmol) of 1,2,4,5-tetramethylimidazo[4,5-*c*]pyridinium iodide and 0.51 mL (4.2 mmol) of *n*-2,5-methoxybenzaldehyde; yield is 0.96 g (63.3%), and m.p. is 235–236°C (H_2O).

Found: N 10.5%.

$C_{18}H_{20}N_3OI$.

Calculated: N 10.0%.

Example 4. 1,5-Dimethyl-2-phenyl-(2,5-dimethoxystyryl)imidazo[4,5-*c*]pyridinium iodide (IYu-428).

[This compound] is obtained by analogy with Example 1, by proceeding from 0.8 g (2.2 mmol) of 1,4,5-trimethyl-2-phenylimidazo[4,5-*c*]pyridinium iodide and 0.546 g (3.3 mmol) of 2,5-dimethoxybenzaldehyde; yield is 0.85 g (71.2%), and m.p. is 233–234°C (*n*-butanol).

Found: N 8.3%.

$C_{24}H_{14}N_3O_2I$.

Calculated: N 8.2%.

Example 5. 1,5-Dimethyl-2-phenyl-4-*n*-N′,N′-dimethylaminostyryl)imidazo[4,5-*c*]pyridinium iodide (IYu-431).

[This compound] is obtained by analogy with Example 1, by proceeding from 0.8 g (2.2 mmol) of 1,4,5-trimethyl-2-phenylimidazo[4,5-*c*]pyridinium iodide and 0.448 g (3 mmol) of *n*-N,N-dimethylaminobenzaldehyde; yield is 0.85 g (78.2%), and m.p. is 265–266°C (*n*-butanol).

Found: C 57.7%, H 5.2%.

$C_{24}H_{25}N_4I$.

Calculated: C 58.0%, H 5.1%.

Fungicidal activity was determined in the mycelium of fungi: *Botrytis cinerea*, *Fusarium moniliforme*, *Venturia inaegualis*, *Aspergillus niger*, and *Verticillium dahlick*,* and in the bacterium *Xanthomonas malvacearum*.

The new substances are dissolved in acetone and introduced, under sterile conditions, into liquefied agar, which is poured into Petri dishes. Tetramethylthiuramdisulfide (TMTD) is taken as the reference. The concentration of the active ingredient is 0.003%. Some 18–20 hr after pouring and solidification, the agar slab is inoculated with pieces of mycelium and the aforementioned test objects, and is held for 4–5 days at a temperature of 22–25°C. At the end of this period, the size of the colonies of the fungi studied is determined, and then Abbott's formula is used to determine the percent suppression (P) of the fungal mycelium in comparison with the reference:

$$P = \frac{a-c}{a} \times 100,$$

where a is the growth of the fungal mycelium in the control, and c is the growth of the fungal mycelium on the preparation.

The test results are presented in the table.

In terms of fungicidal activity, the compounds bearing identifiers IYu-452, IYu-428, IYu-5, and IYu-6 surpass or are equivalent to the reference TMTD on mycelium of the fungus *Verticillium dahlial.* The compounds IYu-6 and IYu-452 also exhibit pronounced bactericidal activity. The compound IYu-5 has activity toward the pathogen of gray mold. In addition to high fungicidal activity, the substances being filed for exhibit high selectivity of action toward fungal diseases, and this in turn protects the environment from pollution due to excessive use of chemicals as means of controlling parasitic organisms.

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*Translator's note: In the Russian authorship certificate this species name is given in two spellings: *Verticillium dahlick* and *Verticillium dahlial.* Both spellings are used in this translation to match the original text.

Test Results for Fungicidal Activity
(The Compounds Were Tested at a Concentration of 0.003%,
Referred to the Active Ingredient)

Identifiers of compounds	*Xanthomonas malvacearum*	*Botrytis cinerra* [*sic*]	*Fusarium moniliforme*	*Venturia inaegualis*	*Aspergillus niger*	*Verticillium dahlial*
IYu-452	75	36	22	25	8	92
IYu-431	12	68	11	6	17	—
IYu-428	12	18	33	25	17	83
IYu-5	50	100	4	14	13	100
IYu-6	100	63	0	31	13	100
TMTD	87	100	100	100	87	83

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(19) **SU** (11) **860,463 A1**

(51) International patent classification:
C 07 D 471/04; A 61 K 31/415, 31/44

(12) DESCRIPTION OF AN INVENTION
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(21) (22) Application: 2,908,344/04, April 9, 1980

(46) Date of publication: May 27, 1998

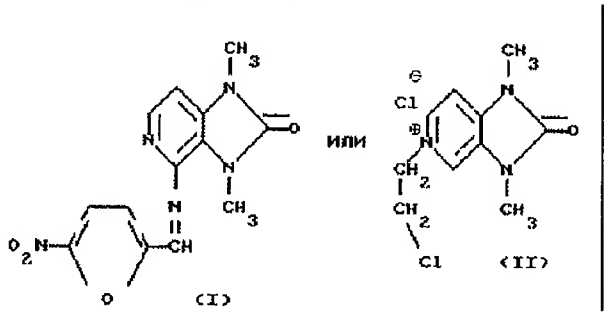
(56) References: 1. Z. Talik and B. Brekiess, "Some pyridotriazoles and imidazoles," *Roczn. Chem.*, 1964, 38(5), p. 887, cited after *Chem. Abs.*, 62, p. 5271. 2. Yu. M. Yutilov, K. M. Khabarov, and I. A. Svertilova, Deposit No. 4182-79, 1979.

(71) Applicants: Institute of Physicoorganic Chemistry and Coal Chemistry of the Ukrainian SSR Academy of Sciences, and the All-Union Scientific Research Institute of Plant-Protection Chemicals.

(72) Inventors: K. M. Khabarov, Yu. M. Yutilov, and V. V. Galitsina.

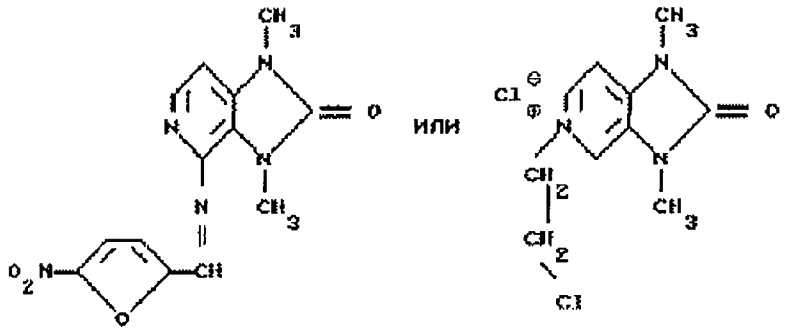
(54) DERIVATIVES OF 4-AMINO-1,3-DIMETHYLIMIDAZO[4,5-*c*]PYRIDIN-2-ONE EXHIBITING ACARICIDAL ACTION

(57) Derivatives of 4-amino-1,3-dimethylimidazo[4,5-*c*]pyridin-2-one having the formula:



which exhibit acaricidal action.

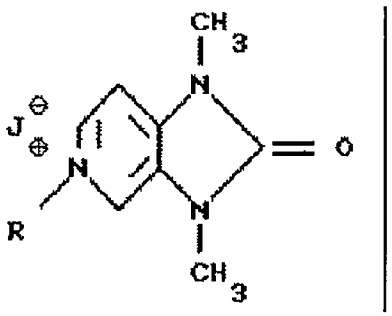
New derivatives of 4-amino-1,3-dimethylimidazo[4,5-*c*]pyridin-2-one having formula I or II are proposed:



which exhibit acaricidal action.

4-Hydrazinoimidazo[4,5-*c*]pyridine, which exhibits hypotensive activity [1], is known.

Also known are quaternary salts of 1,3-dimethyl-4-aminoimidazo[4,5-*c*]pyridin-2-one [2] having the general formula



where R is an alkyl, benzyl, or allyl.

However, the literature contains no data on the activity of these compounds.

The object of the invention is to expand the variety of chemical compounds that act on the living organism.

The stated object is attained by derivatives of 1,3-dimethyl-4-aminoimidazo[4,5-*c*]pyridin-2-one having formulas I and II, which exhibit acaricidal action.

Compound I is obtained by reacting 4-amino-1,3-dimethylimidazo[4,5-*c*]pyridin-2-one with 5-nitrofurfurol in alcohol while it is being boiled.

Compound II is obtained by reacting 4-amino-1,3-dimethylimidazo[4,5-*c*]pyridin-2-one with ethylene chlorohydrin at 170–180°C, followed by treatment with thionyl chloride in chloroform at 50–60°C.

Example 1.

4-(5-Nitrofurfurylidene-2-amino)-1-3-dimethylimidazo[4,5-*c*]pyridin-2-one (I).

A quantity of 0.8 g of 4-amino-1,3-dimethylimidazo[4,5-*c*]pyridin-2-one and 0.65 g of 5-nitrofurfurol are boiled in alcohol for 1 hr and cooled, and the precipitate is filtered off and dried. Yield is 1.12 g, and m.p. is 238–240°C (from ethanol).

Found: C 51.52%, H 4.01%.

$C_{13}H_{11}N_5O_4$.

Calculated: 51.82%, H 3.68%.

Example 2.

4-Amino-5-(2′-chloroethyl)-1,3-dimethylimidazo[4,5-*c*]pyridinium-2-one (II) chloride.

A quantity of 0.99 g of 4-amino-1,3-dimethylimidazo[4,5-*c*]pyridin-2-one in 1.5 mL of ethylene chlorohydrin is heated at 170–180°C for 1 hr, the ethylene chlorohydrin is driven off, 10 mL of dry chloroform and 0.5 mL of thionyl chloride are added, and [the resulting mixture] is heated at 50–60°C for 40 min and cooled, and the precipitate is washed with acetone and dried. Yield is 1.162 g, and m.p. is 172–173°C (from ethanol).

Found: C 42.88%, H 5.19%, Cl 25.93%, N 19.79%.

$C_{10}H_{14}Cl_2N_4O$.

Calculated: C 43.32%, H 5.05%, Cl 25.60%, N 20.22%.

Infrared spectrum: 3465 and 3265 cm^{-1} (ν_{NH_2}), 1705 cm^{-1} ($\nu_{C=O}$).

The compounds obtained are tested on beans for acaricidal activity in the two-spotted spider mite (*Tetranychus urticae* Koch).

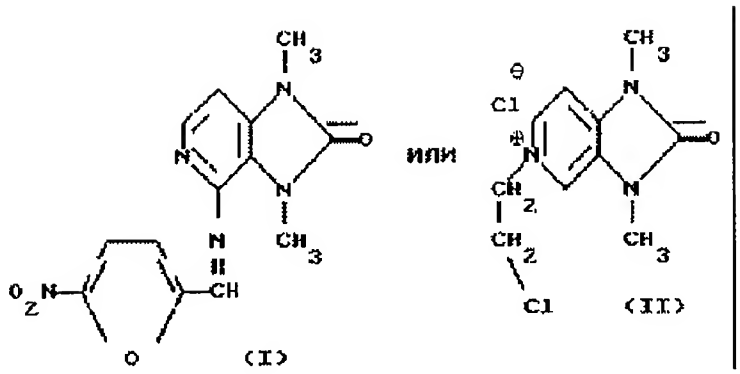
Standard cuttings from bean leaves with adult two-spotted spider mites placed on them are sprinkled with 2.5 mL of a water–acetone solution of the test compound at a concentration of 0.1% active ingredient. After the moisture in liquid drops dries away, the cuttings with the treated mites are placed in a wet chamber. The mortality of the mites is calculated after 48 hr.

Data on the acaricidal activity of the compounds tested are presented in the table.

Thus, the proposed compounds exhibit acaricidal activity and may find broad application in agricultural pest control.

Claim

Derivatives of 4-amino-1,3-dimethylimidazo[4,5-*c*]pyridin-2-one having the formula



which exhibit acaricidal action.

Compound	Mortality (%) of mites from a concentration of 0.1% referred to the active ingredient
4-Amino-5-(2′-chloroethyl)-1,3-dimethylimidazo[4,5-*c*]pyridinium-2-one chloride	84
4-(5-Nitrofurfurylidene-2-amino)-1,3-dimethylimidazo[4,5-*c*]pyridin-2-one	85
Reference: Kelthane	100

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(54) Title: TUMOR ANTIGENS AND CTL CLONES ISOLATED BY A NOVEL PROCEDURE

(57) Abstract

The present invention relates to isolation of cytotoxic T lymphocyte (CTL) clones. In particular, the present invention relates to isolated CTL clones that are specific for proteins of the MAGE family. The CTL clones of the present invention have been isolated by successive steps of stimulation and testing of lymphocytes with antigen presenting cells which present antigens derived from different expression systems, e.g., from recombinant *Yersinia*, recombinant *Salmonella*, or recombinant viruses. The present invention further relates to antigenic peptides as well as the peptide/HLA complexes which are recognized by the isolated CTL clones.

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BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
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CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
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TUMOR ANTIGENS AND CTL CLONES ISOLATED BY A NOVEL PROCEDURE

FIELD OF INVENTION

The present invention relates to isolation of cytotoxic T lymphocyte (CTL) clones. The CTL clones of the present invention have been isolated by successive steps of stimulation and testing of lymphocytes with antigen presenting cells which present antigens derived from different expression systems, e.g., from recombinant *Yersinia*, recombinant *Salmonella*, or recombinant viruses. The present invention further relates to isolated CTL clones that are specific for proteins of the MAGE family. Antigenic peptides as well as the peptide/HLA complexes which are recognized by the isolated CTL clones are also provided.

BACKGROUND

An important facet of the immune response in a mammalian subject is the recognition by T cells of the complexes of the cell surface molecules, i.e., the complexes of peptides and HLA (human leukocyte antigens) or MHC (major histocompatibility complexes) molecules. These peptides are derived from larger molecules which are processed by the cells which also present the HLA/MHC molecules. See in this regard, Male et al., *Advanced Immunology* (J.P. Lipincott Company, 1987), especially chapters 6-10. The interaction between T cell and HLA/peptide complexes is restricted, requiring a T cell specific for a particular combination of an HLA molecule and a peptide. If a specific T cell is not present, there is no T cell response even if its partner complex is present. Similarly, there is no response if the

specific complex is absent, but the T cell is present. This mechanism is involved in the immune system response to foreign materials, in autoimmune pathologies, and in responses to cellular abnormalities.

Most progressively growing neoplastic cells express potentially immunogenic tumor-associated antigens (TAAs), also called tumor rejection antigens (TRAs). A number of genes have been identified that encode tumor rejection antigen precursors (or TRAPs), which are processed into TRAs in tumor cells. Such TRAP-encoding genes include members of the MAGE family, the BAGE family, the DAGE/PRAME family, the GAGE family, the RAGE family, the SMAGE family, NAG, Tyrosinase, Melan-A/MART-1, gp 100, MUC-1, TAG-72, CA125, mutated proto-oncogenes such as praise, mutated tumor suppressor genes such as p53, tumor associated viral antigens such as HPV16 E7. See, e.g., review by Van den Eynde and van der Bruggen (1997) in *Curr. Opin. Immunol.* 9:684-693, Sahin et al. (1997) in *Curr. Opin. Immunol.* 9:709-716, and Shawler et al. (1997) *Advances in Pharmacology 40*: 309-337, Academic Press, Inc., San Diego, California.

TRAs, like other antigenic epitopes, are presented at the surface of tumor cells by MHC molecules and have been shown to induce a CTL response *in vivo* and *in vitro*. See, for example, van der Bruggen et al. (1991) *Science 254*: 1643-1647. However, such TRA-expressing tumor cells do not provoke reliable anti-tumor immune responses *in vivo* that are capable of controlling the growth of malignant cells. Boon et al. (1992) *Cancer Surveys 13*: 23-37; T. Boon (1993) *Int. J. Cancer 54*: 177-180; T. Boon (1992) *Advances Cancer Res. 58*: 177-209. Thus, generation of CTL clones that recognize specific TRAs provides a powerful tool for

tumor therapeutics. The identification of TRAs also allows the design of recombinant vaccines for the treatment of various pathological conditions.

The present invention provides a novel procedure for isolating CTL clones. By following such procedure, novel CTL clones have been isolated that recognize specific antigenic peptides of proteins, preferably of the MAGE family. The MHC molecules presenting these peptides have been identified as well.

SUMMARY OF THE INVENTION

One embodiment of the present invention provides methods for isolating CTL clones from a blood sample.

The methods of the present invention include successive steps of stimulating and testing lymphocytes with antigen presenting cells. Such methods, by employing different antigen presenting cells at different steps, significantly reduce non-specific CTL activities generated in the procedure and permit more efficient isolation of CTL clones.

Antigen presenting cells which are used in the methods of the present invention can differ in cell type and/or in the expression system from which the antigen to be presented is derived. Cells which can be employed as antigen presenting cells in the present methods include professional and facultative antigen presenting cells. A preferred antigen presenting cell is an autologous dendritic cell, an autologous B cell transformed with EBV, or an activated T cell.

Antigen presenting cells can be modified by a variety of ways to effect the expression of an antigen of interest at the cell surface, preferably, by infection

with a recombinant *Yersinia*, recombinant *Salmonella*, or recombinant viruses. Preferred recombinant viruses include vaccinia, canarypox virus, other pox viruses, adenovirus, herpes simplex virus, and retrovirus.

The protein against which CTL clones are generated can be a tumor associated protein, an antigenic protein of a pathogen, or the like. Preferably, the protein is a member of the MAGE family, in particular, MAGE-A1, MAGE-A3 and MAGE-A4.

In another embodiment, the present invention contemplates CTL clones isolated by using the methods of the present invention.

In a preferred embodiment, the present invention provides isolated CTL clones that are specific for peptide/HLA complexes SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48)/HLA-A28, AELVHFLLL (SEQ ID NO: 55)/HLA-B40, and RVRFFFPSL (SEQ ID NO: 57)/HLA-B7, respectively.

In a more preferred embodiment, the present invention provides isolated CTL clones LB1137 462/F3.2, LB1801 456/H7.11, LB1118 466/D3.31, LB 1801 456/H8.33, LB1137 H4.13, LB1841 526/F7.1 and LB1803 483/G8.4.

Furthermore, the present invention provides methods of identifying antigenic peptide epitopes of a protein by using CTL clones isolated following the methods of present invention.

In still another embodiment, the present invention provides newly isolated antigenic peptides, DPARYEFLW (MAGE-A1 258-266) (SEQ ID NO: 42), GVYDGREHTV (MAGE-A4 230-239) (SEQ ID NO: 44), SAFPTTINF(SEQ ID NO: 47) (MAGE-A1 62-70), EVYDGREHSA(SEQ ID NO: 48) (MAGE-A1

222-231), AELVHFLLL (SEQ ID NO: 55) (MAGE-A3 114-122), RVRFFFPSL (SEQ ID NO: 57) (MAGE-A1 289-297). Nucleic acid sequences encoding such peptides are also contemplated.

In another embodiment, the present invention provides isolated peptide/HLA complexes, peptide SAYGEPRKL (SEQ ID NO: 2) complexed with HLA-Cw3, peptide DPARYEFLW(SEQ ID NO: 42) complexed with HLA-B53, peptide GVYDGREHTV(SEQ ID NO: 44) complexed with HLA-A2, peptide SAFPTTINF(SEQ ID NO: 47) complexed with HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48) complexed with HLA-A28, AELVHFLLL (SEQ ID NO: 55) complexed with HLA-B40, and RVRFFFPSL (SEQ ID NO: 57) complexed with HLA-B7.

In another embodiment, cells expressing any of these peptide/HLA complexes are contemplated.

Still another embodiment of the invention provides pharmaceutical compositions which include any one of the isolated CTL clones, the antigenic peptides, the peptide/HLA complexes, and cells expressing the peptide/HLA complexes of the present invention.

In a further aspect, the present invention provides methods useful for diagnosing and treating various pathological conditions.

One embodiment of the present invention provides methods of diagnosing in a subject, a pathological condition characterized by an abnormal expression of a peptide/HLA complex, by detecting the presence of cells abnormally expressing such complex in the subject.

Another embodiment of the present invention provides methods of detecting in a subject, the presence of cells abnormally expressing a peptide/HLA complex of the present invention by using an isolated CTL clone of

the present invention which specifically recognizes such complex.

One embodiment of the present invention provides methods of diagnosing in a subject, a pathological condition characterized by an abnormal expression of a peptide/HLA complex, by detecting an increased frequency of CTL cells specific for such complex.

Another embodiment of the present invention provides methods of detecting in a subject the presence, of CTL cells specific for a peptide/HLA complex of the present invention by using an antigen presenting cell expressing such complex at the cell surface.

In still another embodiment, the present invention provides methods of treating a subject of a pathological condition characterized by an abnormal expression of a peptide/HLA complex of the present invention by administering to the subject, a therapeutically effective amount of cells of a CTL clone specific for such complex.

Another embodiment provides methods of treating a subject of a pathological condition characterized by an abnormal expression of a peptide/HLA complex of the present invention, by administering to the subject a therapeutically effective amount of the peptide.

Still another embodiment provides methods of treating a pathological condition characterized by an abnormal expression of a peptide/HLA complex of the present invention, by obtaining antigen presenting cells from the subject, modifying such cells to effect a presentation of the peptide/HLA complex at the cell surface, and then reperfusing such "loaded" cells into the subject.

BRIEF DESCRIPTION OF FIGURES

Figure 1 illustrates the plasmid map of the expression vector pMS111-MAGE-A1 ($YopE_{130}$-MAGE-A1).

Figure 2 (A) depicts the procedure for stimulating CTL 82/30 with EBV-transformed human B cells (HLA-A1) mixed with recombinant *Yersinia*; (B) depicts the quantitation of IFN-released by activated CTLs.

Figure 3 depicts the specific recognition by CTL clone LB1137 462/F3.2 of a MAGE-A1 antigenic peptide presented by HLA-Cw3.

Figure 4 depicts the specific recognition by CTL clone LB1801 456/H7.11 of a MAGE-A1 antigenic peptide presented by HLA-B53.

Figure 5 depicts the specific recognition by CTL clone LB1137 H4.13 of a MAGE-A4 antigenic peptide presented by HLA-A2.

Figure 6 depicts the MAGE-A4 nucleotide sequences (SEQ ID NO: 49) and the primers used in PCR as described in Example 9.

Figure 7 depicts a MAGE-A1 peptide presented by HLA-Cw2 to CTL clone LB1118 466/D3.31.

7A. Lysis by CTL clone LB1118 466/D3.31 of autologous EBV-B cells infected with vaccinia-MAGE-A1. Target cells were infected for 2 hours at an MOI of 20, ^{51}Cr-labeled, and incubated with CTL clone LB1118 466/D3.31 for 4 hours. Targets infected with the parental vaccinia were used as a negative control.

7B. Stimulation of CTL clone LB1118 466/D3.31 by COS-7 cells that were transiently transfected with a MAGE-A1 cDNA and a cDNA encoding HLA-Cw2. One day after transfection, 1,500 CTL clone LB1118 466/D3.31 were added

into microwells containing $1.5x10^4$ transfected COS-7 cells. TNF production was estimated after overnight coculture by testing the toxicity of the supernatants for the TNF-sensitive cells of WEHI-164 clone 13.

7C. Lysis by CTL clone LB1118 466/D3.31 of autologous EBV-B cells incubated with synthetic peptide SAFPTTINF(SEQ ID NO: 47) (MAGE-A1$_{62-70}$). Targets were ^{52}Cr-labeled and incubated for 4 hours with the CTL, at an effector-to-target ratio of 5:1, in the presence of the peptide at the concentrations indicated.

7D. Lysis of HLA-Cw2 tumor cell lines by CTL clone LB1118 466/D3.31. Target cells were ^{51}Cr-labeled and incubated for 4 hours with CTL clone LB1118 466/D3.31 at various effector-to-target ratios.

Figure 8 depicts a MAGE-A1 peptide presented by HLA-A28 to CTL clone LB1801 456/H8.33.

8A. Lysis by CTL clone LB1801 456/H8.33 of autologous EBV-B cells infected with vaccinia-MAGE-A1. Target cells were infected for 2 hours at an MOI of 20, ^{51}Cr labeled, and incubated with CTL clone LB1801 456/H8.33 for 4 hours. Targets infected with the parental vaccinia were used as a negative control.

8B. Stimulation of CTL clone LB1801 456/H8.33 by COS-7 cells transiently transfected with a MAGE-A1 cDNA and a cDNA encoding HLA-A28. One day after transfection, 1,500 CTL clone LB1801 456/H8.33 were added into microwells containing $1.5x10^4$ transfected COS-7 cells. TNF production was estimated after overnight coculture by testing the toxicity of the supernatants for the TNF-sensitive cells of WEHI-164 clone 13.

8C. Lysis by CTL clone LB1801 456/H8.33 of autologous EBV-B cells incubated with synthetic peptide

EVYDGREHSA(SEQ ID NO: 48) (MAGE-A1 $_{222-231}$). Targets were ^{51}Cr-labeled and incubated for 4 hours with CTL clone LB1801 456/H8.33, at an effector-to-target ratio of 5:1, in the presence of the peptide a the concentrations indicated.

8D. Lysis of HLA-A28 melanoma line by CTL clone LB1801 456/H8.33. Target cells were ^{51}Cr-labeled and incubated for 4 hours with CTL clone LB1801 456/H8.33 at various effector-to-target ratios.

Figure 9. A MAGE-A3 peptide is presented to CTL clone LB1841 526/F7.1 by HLA-B40.

9A. Lysis by CTL clone LB1841 526/F7.1 of autologous EBV-B cells infected with vaccinia-MAGE-A3.

9B. Stimulation of CTL clone LB1841 526/F7.1 by COS-7 cells transiently transfected with a MAGE-A3 cDNA and a cDNA encoding an HLA molecule as indicated.

9C. Lysis by CTL clone LB1841 526/F7.1 of autologous EBV-B cells incubated with synthetic peptide AELVHFLLL (SEQ ID NO: 55) (MAGE-A3 $_{114-122}$).

9D. Lysis of HLA-B40 melanoma cells by CTL clone LB1841 526/F7.1.

Figure 10. A MAGE-A1 peptide is presented to CTL clone LB1803 483/G8.4 by HLA-B7.

10A. Lysis by CTL clone LB1803 483/G8.4 of autologous EBV-B cells infected with vaccinia-MAGE-A1.

10B. Stimulation of CTL clone LB1803 483/G8.4 by COS cells transiently transfected with a MAGE-A1 cDNA and a cDNA encoding an HLA molecule as indicated.

10C. Lysis by CTL clone LB1803 483/G8.4 of autologous EBV-B cells incubated with synthetic peptide RVRFFFPSL (SEQ ID NO: 57) (MAGE-A1 $_{289-297}$).

10D. Lysis of HLA-B7 melanoma cells by CTL clone LB1803 483/G8.4.

DETAILED DESCRIPTION OF THE INVENTION

One embodiment of the present invention provides novel methods for isolating CTL clones. The present methods include successive steps of stimulating and testing lymphocytes by using different antigen presenting cells at different steps.

The procedure to develop specific CTL clones *in vitro* has been described. Briefly, a blood sample containing T-cell precursors is taken from a mammal. PBLs are purified from such blood sample and are incubated with stimulator cells which express antigenic peptides complexed with the appropriate MHC molecule. Stimulator cells can be tumor cells (see, e.g., the United States Patent No. 5,342,774, Knuth et al. (*Proc. Natl. Acad. Sci. USA* 86: 2804-2808, 1989) and Van Den Eynde et al. (*Int. J. Cancer* 44: 634-640, 1989), or antigen presenting cells pulsed with defined peptides. Additional components, e.g., allogeneic feeder cells and cytokines, can be added into the incubation mixture. CTLs specific for antigens expressed at the surface of the stimulator cells will proliferate, and thus, will be enriched in the cell population as a result of the stimulation. CTL clones can be subsequently isolated by, e.g., limiting dilution. However, the approach using antigen presenting cells pulsed with defined peptides as stimulator cells, have sometimes generated CTLs that are unable to recognize the relevant tumor cells.

The present inventors have found that efficient isolation of CTL clones can be achieved by successive steps of stimulating and testing T cell precursors, using

different antigen presenting cells at different steps. The present methods of isolating CTL clones permit significant reduction of CTL activities generated toward non-specific molecules, e.g., molecules expressed from the backbone sequence of an expression vector.

By "different antigen presenting cells" it means that the antigen presenting cells may differ in cell type or in the expression system from which an antigen of interest being presented is derived.

"Antigen presenting cells" as referred herein, express at least one class I or class II MHC determinant and may include those cells which are known as professional antigen-presenting cells such as macrophages, dendritic cells and B cells. Other professional antigen-presenting cells include monocytes, marginal zone Kupffer cells, microglia, Langerhans' cells, interdigitating dendritic cells, follicular dendritic cells, and T cells. Facultative antigen-presenting cells can also be used according to the present invention. Examples of facultative antigen-presenting cells include activated T cells, astrocytes, follicular cells, endothelium and fibroblasts. As used herein, "antigen-presenting cells" encompass both professional and facultative types of antigen-presenting cells.

The antigen presenting cells can be isolated from tissue or blood samples (containing peripheral blood mononuclear cells) obtained from a mammal such as human. Cell lines established from such samples may also be used. Procedures for establishing cell lines are well known in the art. Certain cell lines may be obtained directly from the American Type Culture Collection, 12301

Parklawn Drive, Rockville, Maryland, 20852-1776. Both normal and malignant cells can be employed.

Preferably, the MHC determinants expressed by the antigen presenting cells are compatible with those expressed by the mammal from which the sample containing T cell precursors is taken. More preferably, autologous antigen presenting cells or cell lines established therefrom are employed. Non-autologous cells may be used as long as the MHC determinants expressed by such cells are compatible, either naturally, by way of transfection or other means that are appropriate. One skilled in the art is also familiar with the methods for determining whether the MHC molecules expressed by an antigen presenting cell are compatible with those of the mammal subject involved, such as well known HLA-typing procedures. See general teachings by Coligan et al. (1994) *Current Protocols in Immunology* John Wiley & Sons Inc: New York, New York.

Preferred antigen presenting cells are autologous dendritic cells, autologous B cells transformed with EBV, and autologous T cell activated by PHA.

Further, according to the present invention, antigen presenting cells used in the present methods can also differ in the expression system from which an antigen of interest is derived. More specifically, the antigen presenting cells can be modified in various ways to effect the expression of an antigen at the cell surface. For example, an antigen presenting cell can be infected with a recombinant *Yersinia*, a recombinant *Salmonella*, or a recombinant virus. In each case, the recombinant microorganism encodes a protein from which the peptide antigen presented is derived.

The protein expressed from any of these expression systems is processed in the antigen presenting cells into small peptides, which are then complexed with the appropriate MHC molecules and presented at the cell surface. In the present invention, peptides that are complexed with MHC molecules and presented at the cell surface are also referred to as "antigens".

The term "*Yersinia*" as used herein includes all species of *Yersinia*, including *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Yersinia pestis*. The term "recombinant *Yersinia*" used herein refers to *Yersinia* genetically transformed with an expression vector. The term "delivery" used herein refers to the transportation of a protein from a *Yersinia* to an antigen presenting cell, including the steps of expressing the protein in the *Yersinia*, secreting the expressed protein(s) from such *Yersinia* and translocating the secreted protein(s) by such *Yersinia* into the cytosol of the antigen presenting cell.

According to the present invention, preferred *Yersinia* for use in expressing and delivering the protein of interest are mutant *Yersinia* that are deficient in producing functional effector proteins.

A preferred mutant *Yersinia* strain for use in expressing and delivering the protein of interest is a quintuple-mutant *Yersinia* strain in which all the effector-encoding genes are mutated such that the resulting *Yersinia* no longer produce any functional effector proteins. Such quintuple-mutant *Yersinia* strain is designated as *yopEHOMP* for *Y. enterocolitica* or *yopEHAMJ* for *Y. pseudotuberculosis*. One example of such *yopEHOMP* strain is *Y. enterocolitica* MRS40(pABL403).

An antigenic protein of interest can be cloned into a yersinia expression vector Ffr used in combination with a mutant *Yersinia* for delivery of the protein into antigen presenting cells. In accordance with the present invention, such a vector is characterized by (in the 5' to 3' direction) a promoter, a first nucleic acid sequence encoding a delivery signal, a second nucleic acid sequence fused thereto coding for the protein to be delivered and other sequences that may be appropriate (e.g., a polyadenylation signal).

The promoter of the expression vector is preferably from a *Yersinia* virulon gene. A "*Yersinia* virulon gene" refers to genes on the *Yersinia* pYV plasmid, the expression of which is controlled both by temperature and by contact with a target cell. See review by Cornelis et al. (1997). Such genes include genes coding for elements of the secretion machinery (the *Ysc* genes), genes coding for translocators (*YopB*, *YopD*, and *LcrV*), genes coding for the control elements (*YopN* and *LcrG*), and genes coding for effectors (*YopE*, *YopH*, *YopO/YpkA*, *YopM* and *YopP/YopJ*). Preferably, the promoter is from an effector-encoding gene selected from any one of *YopE*, *YopH*, *YopO/YpkA*, *YopM* and *YopP/YopJ*. More preferably, the promoter is from *YopE*.

Further, in accordance with the present invention, a first DNA sequence coding for a delivery signal is operably linked to the promoter. "A delivery signal", as described hereinabove, refers to a polypeptide which can be recognized by the secretion and translocation system of *Yersinia* and therefore directs the secretion and translocation of a protein into a an antigen presenting cell. Such polypeptide is from an effector protein including YopE, YopH, YopO/YpkA, YopM,

and YopP/YopJ, and preferably, YopE. More preferably, the effector protein is YopE of *Yersinia enterocolitica*.

One skilled in the art is familiar with the methods for identifying the polypeptide sequences of an effector protein that are capable of delivering a protein. For example, one such method is described by Sory et al. (1994). Examples of such delivery signal polypeptides include from *Y. enterocolitica*: $YopE_{130}$ (the N-terminal 130 amino acids of YopE), $YopE_{50}$, $YopM_{100}$ and $YopH_{71}$.

The yersinia expression vectors may be transformed into *Yersinia* by a number of known methods which include, but are not limited to, electroporation, calcium phosphate mediated transformation, conjugation, or combinations thereof. For example, a vector can be transformed into a first bacteria strain by a standard electroporation procedure. Subsequently, such a vector can be transferred from the first bacteria strain into *Yersinia* by conjugation, a process also called "mobilization". *Yersinia* transformant (i.e., *Yersinia* having taken up the vector) may be selected, e.g., with antibiotics. These techniques are well known in the art. See, for example, Sory et al. (1994).

The delivery of a protein from a recombinant Yersinia into the cytosol of an antigen presenting cell can be effected by contacting an antigen presenting cell with a recombinant *Yersinia* under appropriate conditions. Multiple references and techniques are available for those skilled in the art regarding the conditions for inducing the expression and translocation of virulon genes, including the desired temperature, Ca++ concentration, manners in which *Yersinia* and target cells are mixed, and the like. See, for example, Cornelis,

Cross talk between Yersinia and eukaryotic cells, Society for General Microbiology Symposium, 55; Mocrae, Saunders, Smyth, Stow (eds), *Molecular aspects of host-pathogen interactions*, Cambridge University Press, 1997. The conditions may vary depending on the type of eukaryotic cells to be targeted, e.g.: the conditions for targeting human epithelial carcinoma Hela cells (Sory et al. (1994)); the conditions for targeting mouse thymoma or melanoma cells (Starnbach et al. (1994) *J. Immunol. 153*: 1603); and the conditions for targeting mouse macrophages (Boland et al. (1996)). Such variations can be addressed by those skilled in the art using conventional techniques.

Those skilled in the art can also use a number of assays to determine whether the delivery of a fusion protein is successful. For example, the fusion protein may be labeled with an isotope or an immunofluoresceine, or detected by a immunofluoresceine conjugated antibody, as disclosed by Rosqvist et al. (1994) *EMBO J. 13*: 964. The determination can also be based on the enzymatic activity of the protein being delivered, e.g., the assay described by Sory et al. (1994). The determination can also be based on the antigenicity of the protein being delivered. For example, the delivery of a MAGE-A1 protein into EBV-transformed human B cells can be detected by the recognition of such targeted B cells by CTL cells specific for MAGE-A1 epitopes. Such CTL recognition, in turn, may be detected by a number of assays including assaying the secretion of IFN-γ from the activated CTLs or Cr^{51} release from lysed target cells. Methods such as Western-blot analysis using antibodies specific against the protein being delivered, PCR in situ hybridization, or ELISPOT (Mabtech AB, Sweden) may also

be employed for such determination. See, e.g., W. Herr et al. (1997) *J. Immunol. Methods 203*: 141-152 and W. Herr et al. (1996) *J. Immunol. Methods 191*: 131-142.

In accordance with the present invention, the antigenic protein of interest can also be expressed from a recombinant Salmonella. For example, avirulent strains of *Salmonella typhimurium* can be used as antigen delivery vectors. It is known in the art that antigenic epitopes, such as viral epitopes can be successfully delivered to the host cell cytosol by using the type III protein secretion system of *S. typhimurium*. See, e.g., Russmann et al. (1998) 281: 565-568.

In accordance with the present invention, the expression of a protein of interest in the antigen presenting cell can also be effected by infection of the antigen presenting cells with a recombinant virus. In particular, the present invention contemplates recombinant viruses of vaccinia, canarypox virus, other pox viruses, adenovirus, herpes simplex virus, retrovirus and any other viruses that are appropriate.

A preferred strain of vaccinia for use in the present invention is the WR strain (Panicali et al.(1981), *J. Virol.* 37: 1000-1010). The nucleotide sequence coding for the protein of interest can be operably linked to a promoter, such as an vaccinia promoter H6, and inserted into a vaccinia vector, thereby generating a donor plasmid. Vaccinia vectors which can be employed for generating adeno-plasmids are available to those skilled in the art and are described in, e.g., U.S. Patent No. 4,769,330. Recombinant WR strains of vaccinia can be generated by using a donor plasmid via *in vivo* recombination, following well-known procedures.

See, e.g., Perkins et al., *J. Virol.* 63: 3829-3936 (1989).

A preferred strain of canarypox virus for use in the present invention is ALVAC (Cox et al. (1993), *Virology* 195: 845-850). The nucleotide sequence coding for the protein of interest can be operably linked to a promoter, such as an vaccinia promoter H6, and inserted into an ALVAC vector to create a donor plasmid. Multiple ALVAC vectors are available to one skilled in the art and are described by, e.g., U.S. Patent No. 5,756,106; Cox et al. (1993) *Virology 195*: 845-850; Tartaglia et al. (1993) *J. Virology 67*: 2370-2375; and Taylor et al. (1992) *Virology 187*: 321-328. Such donor plasmid can be used to generate recombinant ALVAC viruses via in vivo recombination. See, e.g., Cox. et al. (1993); Tartaglia et al. (1993) and Taylor et al. (1992).

Those skilled in the art can also generate recombinant adenoviruses for expressing the protein of interest as described in, e.g., Example 5 hereinafter.

A nucleotide sequence encoding the antigenic protein of interest can be cloned into the various expression vectors as described above. There is no particular limitation in the protein that can be employed in the instant methods for isolating CTL clones.

The term "protein" as used herein refers to naturally occurring proteins as well as artificially engineered proteins, or parts thereof. The term "part of a protein" includes a peptide fragment of a protein that is of sufficient length to be antigenic. Preferably, such a fragment consists of at least 8 or 9 amino acids. "Artificially engineered proteins" as used herein refer to non-naturally occurring proteins, e.g., modified forms of non-naturally occurring proteins, or fusion of two or

more naturally occurring proteins or parts thereof, which are also referred to as polytopes (in-frame fusion of two or more epitopes) as exemplified by Thompson et al. (1995) in *Proc. Natl. Acad. Sci. USA* 92: 5845-5849.

The present invention contemplates, in particular, tumor associated proteins or pathogen associated antigens.

A "tumor associated protein" refers to a protein that is specifically expressed in tumors or expressed at an abnormal level in tumors relative to normal tissues. Such tumor associated proteins include, but are not limited to, members of the MAGE family, the BAGE family (such as BAGE-1), the DAGE/PRAME family (such as DAGE-1), the GAGE family, the RAGE family (such as RAGE-1), the SMAGE family, NAG, Tyrosinase, Melan-A/ MART-1, gp100, MUC-1, TAG-72, CA125, mutated proto-oncogenes such as p21ras, mutated tumor suppressor genes such as p53, tumor associated viral antigens (e.g., HPV16 E7), the SSX family, HOM-MEL-55, NY-COL-2, HOM-HD-397, HOM-RCC-1.14, HOM-HD-21, HOM-NSCLC-11, HOM-MEL-2.4, HOM-TES-11, RCC-3.1.3, NY-ESO-1, and the SCP family. Members of the MAGE family include, but are not limited to, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A11. Members of the GAGE family include, but are not limited to, GAGE-1, GAGE-6. See, e.g., review by Van den Eynde and van der Bruggen (1997) in *Curr. Opin. Immunol.* 9: 684-693, Sahin et al. (1997) in *Curr. Opin. Immunol.* 9: 709-716, and Shawler et al. (1997). These proteins have been shown to associate with certain tumors such as melanoma, lung cancer, prostate cancer, breast cancer, renal cancer and others.

A number of known antigenic proteins from pathogens are also contemplated by the present invention.

The pathogens can include viruses, bacteria, parasites and fungi. Specific examples of antigenic proteins characteristic of a pathogen include the influenza virus nucleoprotein (residues 218-226, as set forth in F. et al. (1997) *J. Virol. 71*: 2715-2721) antigens from Sendai virus and lymphocytic choriomeningitis virus (see, An et al. (1997) *J. Virol. 71*: 2292-2302), the B1 protein of hepatitis C virus (Bruna-Romero et al. (1997) *Hepatology 25*: 470-477), the virus envelope glycoprotein gp 160 of HIV (Achour et al. (1996) *J. Virol. 70*: 6741-6750), amino acids 252-260 or the circumsporozite protein of *Plasmodium berghei* (Allsopp et al. (1996) *Eur. J. Immunol. 26*: 1951-1958), the influenza A virus nucleoprotein (residues 366-374, Nomura et al. (1996) *J. Immunol. Methods 193*: 4149), the listeriolysin O protein of *Listeria monocytogenes* (residues 91-99, An et al. (1996) *Infect. Immun. 64*: 1685-1693), the E6 protein (residues 131-140, Gao et al. (1995) *J. Immunol. 155*: 5519-5526) and E7 protein (residues 21-28 and 48-55, Bauer et al. (1995) *Scand. J. Immunol. 42*: 317-323) of human papillomavirus type 16, the M2 protein of respiratory syncytial virus (residues 82-90 and 81-95, Hsu et al. (1995) *Immunology 85*: 347- 350), the herpes simplex virus type 1 ribonucleotide reductase (see, Salvucci et al. (1995) *J. Gen. Virol. 69*: 1122-1131) and the rotavirus VP7 protein (see, Franco et al. (1993) *J. Gen. Virol. 74*: 2579-2586), *P. falciparum* antigens (causing malaria) and hepatitis B surface antigen (Gilbert et al. (1997) *Nature Biotech.* 15: 1280-1283).

A number of short antigenic peptides can also be employed in the present invention. One skilled in the art can readily determine the length of the fragments

required to produce immunogenic peptides. Alternatively, the skilled artisan can also use coding sequences for peptides that are known to elicit specific T cell responses (either $CD4^+$ or $CD8^+$ T cells), such as tumor-associated antigenic peptides (TAA, also known as TRAs for tumor rejection antigens) as disclosed by U.S. Patent No. 5,462,871, U.S. Patent No. 5,558,995, U.S. Patent No. 5,554,724, U.S. Patent No. 5,585,461, U.S. Patent No. 5,591,430, U.S. Patent No. 5,554,506, U.S. Patent No. 5,487,974, U.S. Patent No. 5,530,096, U.S. Patent No. 5,519,117. Examples of TRAs are provided in Table 1. See also review by Van den Eynde and van der Bruggen (1997) and Shawler et al. (1997). Antigenic peptides of a pathogen origin can also be used, such as those disclosed by Gilbert et al. (1997).

Table 1: Exemplary Antigens

Gene	MHC	Peptide	Position	SEQ ID NO:
MAGE-A1	HLA-A1	EADPTGHSY	161-169	1
	HLA-Cw16	SAYGEPRKL	230-238	2
MAGE-A3	HLA-A1	EVDPIGHLY	168-176	3
	HLA-A2	FLWGPRALV	271-279	4
	HLA-B44	MEVDPIGHLY	167-176	5
BAGE	HLA-Cw16	AARAVFLAL	2-10	6
GAGE-1,2	HLA-Cw16	YRPRPRRY	9-16	7
RAGE	HLA-B7	SPSSNRIRNT	11-20	8
GnT-V	HLA-A2	VLPDVFIRC(V)	2-10/11	9
MUM-1	HLA-B44	EEKLIVVLF	exon 2/ intron	10

		EEKLSVVLF (wild type)		11
CDK4	HLA-A2	ACDPHSGHFV	23-32	12
		ARDPHSGHFV (wild type)		13
β-catenin	HLA-A24	SYLDSGIHF	29-37	14
		SYLDSGIHS (wild type)		15
Tyrosinase	HLA-A2	MLLAVLYCL	1-9	16
	HLA-A2	YMNGTMSQV	369-377	17
	HLA-A2	YMDGTMSQV	369-377	18
	HLA-A24	AFLPWHRLF	206-214	19
	HLA-B44	SEIWRDIDF	192-200	20
	HLA-B44	YEIWRDIDF	192-200	21
	HLA-DR4	QNILLSNAPLGPQFP	56-70	22
	HLA-DR4	DYSYLQDSDPDSFQD	448-462	23
Melan-A^{MART-1}	HLA-A2	(E)AAGIGILTV	26/27-35	24
	HLA-A2	ILTVILGVL	32-40	25
gp100^{Pmel117}	HLA-A2	KTWGQYWQV	154-162	26
	HLA-A2	ITDQVPFSV	209-217	27
	HLA-A2	YLEPGPVTA	280-288	28
	HLA-A2	LLDGTATLRL	457-466	29
	HLA-A2	VLYRYGSFSV	476-485	30
DAGE	HLA-A24	LYVDSLFFL	301-309	31
MAGE-A6	HLA-Cw16	KISGGPRISYPL	292-303	32

As described herein above, sequences coding for a full-length naturally occurring protein, a part of a naturally occurring protein, combinations of parts of a naturally occurring protein, or combinations of different naturally occurring proteins or parts from different

proteins, may all be employed to be cloned into the expression vectors as described hereinabove.

The present invention further provides recombinant expression vectors which can be employed in the present methods, including recombinant yersinia expression vectors, e.g., pMS111-$YopE_{130}$-MAGE-A1 and PMS111-$YopE_{130}$-MAGE-A4; recombinant vaccinia vectors, e.g., WR-MAGE-A1 and WR-MAGE-A4; recombinant canarypox viral vectors, e.g., ALVAC-MAGE-A1; recombinant adenoviral vectors, e.g., adeno-MAGE-A4; and retroviral vectors, e.g., M1-CSM.

To carry out the methods of the present invention, a sample containing T-cell precursors is obtained from a subject, typically, a blood sample from a human subject. The subject can be a cancer patient or an individual without cancer. The sample may be treated to concentrate T-cell precursors prior to stimulation.

The sample is contacted with a first antigen presenting cell expressing a protein, along with any other materials that may be appropriate, such as lymphokines. Upon contact, specific T-cell precursors are activated and begin to proliferate.

Cells in the sample are subsequently tested by contacting the cells with a second antigen presenting cell expressing the protein. The sample can be first diluted and distributed into microwells such that individual cells can be separately tested. CTL Cells which are specific for the protein, or "responding CTLs", can be identified and selected by a variety of standard assays such as a ^{51}Cr release assay, a IFN-γ secretion assay, or a TNF production assay.

In a preferred embodiment of the present invention, the CTL cells thus selected are subject to at

least one additional cycle of stimulation and testing steps.

According to the present invention, the antigen presenting cells used at one step can differ from the cells used in a subsequent step, either in cell type or in the expression system from which the protein is expressed.

For testing the specificity of CTL responses after stimulation, antigen presenting cells of a type that expresses high amounts of class I HLA molecules are preferred, e.g., EBV-transformed B cells.

In a preferred embodiment of the present invention, one of the expression systems used by the antigen presenting cell at one step (either stimulation or testing), is different from at least one of the other expression systems used in another step.

More Preferably, the antigen presenting cells used at a stimulation step employ an expression system different from that used in the immediately following testing step.

The present invention provides examples of combinations of different antigen presenting cells which can be used for isolating specific CTL clones. According to the present invention, $CD8^+$ T lymphocytes obtained from an individual can be stimulated in microwells with autologous monocyte-derived dendritic cells infected with a recombinant ALVAC canarypoxvirus encoding a protein of interest. After several times of stimulation, an aliquot of each microculture can then be tested for specific lysis of autologous EBV-B cells infected with a recombinant Vaccinia encoding the protein of interest. The positive microcultures can then be diluted and stimulated again with autologous EBV-B cells infected

with a recombinant *Yersinia* encoding the protein of interest. Specific clones can be detected and thus isolated by testing for specific lysis of autologous EBV-B cells infected with a recombinant Vaccinia encoding the protein of interest. Thus, the combination of antigen presenting cells used in the foregoing procedure can be characterized as dendritic-ALVAC/EBV-B-Vaccinia/EBV-B-Yersinia/EBV-B-Vaccinia. Additional preferred combinations of antigen presenting cells which can be used in the present methods include: dendritic-Adeno/EBV-B-Vaccinia/EBV-B-Yersinia/EBV-B-Vaccinia, dendritic-ALVAC/EBV-B-Vaccinia/T cell-retroviral /EBV-B-Vaccinia. The present invention is not limited to the above exemplified combinations.

In a further aspect of the invention, the present invention contemplates CTL clones isolated by using the methods of the present invention.

In another embodiment, the present invention contemplates methods for identifying antigenic peptide epitopes of a protein. According to such method, CTL clones that recognize certain antigenic epitopes of a protein are isolated using the present method of isolating CTL clones, as described hereinabove. Such clones can then be used to identify the specific antigenic peptides as well as the presenting HLA molecules, using a variety of well-known procedures, for example, procedures described in Examples 7-11.

According to the methods of the present invention, the identification of an antigenic peptide epitope of a protein is based on the capacity of the peptide/HLA complex, at the surface of an antigen presenting cell, to activate the specific CTLs. The antigenic peptide epitopes thus identified likely

represent the epitopes that are well processed and adequately expressed at the cell surface *in vivo*. By using such method of the present invention, antigenic peptide epitopes from proteins of the MAGE family have been identified; namely, MAGE-A1 peptide 230-238 (presented by HLA-Cw3 and recognized by clone LB1137 462/F3.2), MAGE-A1 peptide 258-266 (presented by HLA-B53 and recognized by clone LB1801 456/H7.11), MAGE-A1 peptide 62-70 (presented by HLA-Cw2 and recognized by clone LB 1118 466/D3.31), MAGE-A1 peptide 222-231 (presented by HLA-A28 and recognized by clone LB1801 456/H8.33), MAGE-A1 peptide 289-297 (presented by HLA-B7 and recognized by clone LB1803 483/G8.4), a MAGE-A3 peptide 114-122 (presented by HLA-B40 and recognized by clone LB1841 526/F7.1), and a MAGE-A4 peptide 230-239 (presented by HLA-A2 and recognized by clone LB1137 H4.13). See **Table 2**. Among these, MAGE-A1 peptide 230-238 (SAYGEPRKL (SEQ ID NO: 2)) has been previously identified, but was found therein to be presented by a different HLA molecule, HLA-Cw16 (U.S. Patent No. 5,558,995).

TABLE 2

GENE	POSITION	PEPTIDE	MHC	CTL	SEQ ID
MAGE-A1	230-238	SAYGEPRKL	HLA-Cw3	LB1137 462/F3.2	2
MAGE-A1	258-266	DPARYEFLW	HLA-B53	LB1801 456/H7.11	42
MAGE-A4	230-239	GVYDGREHTV	HLA-A2	LB1137 H4.13	44
MAGE-A1	62-70	SAFPTTINF	HLA-Cw2	LB1118 466/D3.31	47
MAGE-A1	222-231	EVYDGREHSA	HLA-A28	LB1801 456/H8.33	48
MAGE-A3	114-122	AELVHFLLL	HLA-B40	LB1841 526/F7.1	55
MAGE-A1	289-297	RVRFFFPSL	HLA-B7	LB1803 483/G8.4	57

Accordingly, another embodiment of the present invention provides isolated CTL clones that are specific for peptide/HLA complexes SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48)/HLA-A28, AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and RVRFFFPSL(SEQ ID NO: 57)/HLA-B7, respectively.

In a preferred embodiment, the present invention provides isolated CTL clones LB1137 462/F3.2, LB1801 456/H7.11, LB1118 466/D3.31, LB1801 456/H8.33, LB1137 H4.13, LB1841 526/F7.1 and LB1803 483/G8.4.

In another embodiment, the present invention is directed to the newly isolated antigenic peptides, namely, DPARYEFLW(SEQ ID NO: 42) (MAGE-A1 258-266), GVYDGREHTV(SEQ ID NO: 44) (MAGE-A4 230-239), SAFPTTINF(SEQ ID NO: 47) (MAGE-A1 62-70), EVYDGREHSA(SEQ ID NO: 48) (MAGE-A1 222-231), AELVHFLLL (SEQ ID NO: 55) (MAGE-A3 114-122) and RVRFFFPSL (SEQ ID NO: 57) (MAGE-A1 289-297). Nucleic acid sequences encoding these peptides are also contemplated.

Another embodiment of the present invention is directed to the isolated peptide/HLA complexes of the present invention. Specifically, the present invention provides isolated complex of peptide SAYGEPRKL(SEQ ID NO: 2) and HLA-Cw3, complex of peptide DPARYEFLW(SEQ ID NO: 42) and HLA-B53, complex of peptide GVYDGREHTV(SEQ ID NO: 44) and HLA-A2, complex of peptide SAFPTTINF(SEQ ID NO: 47) and HLA-Cw2, complex of peptide EVYDGREHSA(SEQ ID NO: 48) and HLA-A28, complex of AELVHFLLL (SEQ ID NO: 55) and HLA-B40, and complex of RVRFFFPSL (SEQ ID NO: 57) and HLA-B7.

Once the presenting HLA molecule for an antigenic peptide epitope has been ascertained, a complex of the peptide and the HLA molecule can be made by a variety of methods. For example, the HLA molecule can be produced and isolated by any appropriate recombinant expression system, e.g., an *E. coli*-based expression system. Peptides can be made by, e.g., chemical synthesis or recombinant expression. The peptides and the HLA molecules can then be mixed *in vitro* under conditions that favor the formation of the HLA/peptide complexes. Such conditions are well known in the art. See, e.g., Garboczi et al. (*Proc. Natl. Acad. Sci. USA* 89: 3429-3433, 1992 and Altman et al. (*Science* 274: 94-96, 1996).

The present invention further contemplates cells expressing any of the instant peptide/HLA complexes at the cell surface. Such cells can be made using any antigen presenting cells that are appropriate including cell lines (e.g., COS cells, CHO cells and the like), and by, e.g., peptide loading, or cotransfection as described in the Examples of the present disclosure.

In another embodiment, the present invention contemplates pharmaceutical compositions which include any one of the isolated CTL clones, the isolated antigenic peptides, the isolated peptide/HLA complexes, the antigen presenting cells expressing peptide/HLA complexes of the present invention, or combinations thereof.

The pharmaceutical compositions of the present invention can include other substances such as cytokines, adjuvants and pharmaceutically acceptable carriers. As used herein, a therapeutically acceptable carrier includes any and all solvents, including water,

dispersion media, culture from cell media, isotonic agents and the like that are non-toxic to the host. Preferably, it is an aqueous isotonic buffered solution with a pH of around 7.0. The use of such media and agents in therapeutic compositions is well known in the art. Supplementary active ingredients can also be incorporated into the compositions.

In a further aspect of the present invention, the isolated CTL clones, the isolated antigenic peptides, the cells expressing the peptide/HLA complexes of the present invention are employed in various methods for diagnosing a pathological condition in a subject, preferably, a human subject.

The pathological conditions contemplated by the present invention include tumors and infections by pathogens such as bacteria, parasites, fungus or virus, and the like.

The term "abnormal expression" as used herein refers to an expression that is not present in normal cells or an expression that is present in normal cells at a significantly lower level. In the present invention, "an abnormal expression" can also be used to refer to an unusual processing of a protein which gives rise to an antigenic epitope that is not presented at the surface of normal cells.

In one embodiment, the present invention provides methods of diagnosing in a subject, a pathological condition characterized by an abnormal expression a peptide/HLA complex, by detecting in the subject, the presence of cells abnormally expressing such complex.

In another embodiment, the present invention provides methods of detecting in a subject the presence

of cells abnormally expressing a peptide/HLA complex of the present invention, by using an isolated CTL clone specific for such complex.

According to the present invention, a sample containing the cells suspected to be abnormal is obtained from the subject by, e.g., tissue biopsy. The sample is then contacted with a CTL clone of the present invention. The presence of the abnormal cells can be determined by measuring the activity of the CTL clone (i.e., CTL response) using standard assays such as ^{51}Cr release, IFN-gamma secretion, or TNF production.

In another embodiment, the present invention provides methods for detecting in a subject, the presence of CTL cells specific for an isolated peptide/HLA complex of the present invention. More specifically, a blood sample is secured from the subject and contacted with cells expressing the specific peptide/HLA complexes. The presence of CTL cells specific for the complex can be detected by any of the approaches described hereinabove, e.g., the lysis of the cells expressing the specific peptide/HLA complexes measurable by a standard ^{51}Cr release assay.

Furthermore, the frequency of CTLs specific for a peptide/HLA complex can be assessed by, e.g., limiting dilution analysis or tetramer assays. By comparing with a normal individual, an increased frequency of CTLs specific for a peptide/HLA complex in an individual, is indicative of a pathological condition characterized by an abnormal expression of the complex. Accordingly, the present invention contemplates methods of diagnosing a pathological condition characterized by an abnormal expression of a peptide/HLA complex by detecting an

increased frequency of CTLs specific for such peptide/HLA complex.

In a further aspect of the present invention, the isolated CTL clones, the isolated antigenic peptides, the cells expressing the peptide/HLA complexes of the present invention are employed in various methods for treating a pathological condition in a subject, preferably, a human subject.

The term "treating" is used to refer to alleviating or inhibiting a pathological condition, e.g., inhibiting tumor growth or metastasis, reducing the size of a tumor, or diminishing symptoms of a pathogen infection, by e.g., eliciting an immune response.

In one embodiment, an isolated CTL clone of the present invention can be administered, in a therapy regimen of adoptive transfer, to a subject suffering a pathological condition characterized by an abnormal expression of the peptide/HLA complex that is specifically recognized by such CTL clone. See teachings by Greenberg (1986) *J. Immunol. 136 (5)*: 1917; Riddel et al. (1992) *Science 257*: 238; Lynch et al. (1991) *Eur. J. Immunol. 21*: 1403; and Kast et al. (1989) *Cell 59*: 603 for adoptive transfer. CTLs, by lysing the cells abnormally expressing such antigens, can alleviate or treat the pathological condition at issue, such as a tumor and an infection with a parasite or a virus.

In another embodiment, the present invention provides methods of treating a subject suffering a pathological condition characterized by an abnormal expression of a peptide/HLA complex, by administering the isolated peptides, or the peptide/HLA complexes, to the subject. The pathological condition can be alleviated

by, e.g., specific immune responses elicited due to the administered peptides or peptide/HLA complexes.

In another embodiment of the present invention, a subject suffering a pathological condition characterized by an abnormal expression of a peptide/HLA complex of the present invention, can be treated by obtaining antigen presenting cells from the subject, modifying such cells to effect a presentation of the peptide/HLA complex at the cell surface, and then reperfusing such "loaded" cells into the subject. The modification can be achieved by transfecting the isolated antigen presenting cells with any appropriate expression vectors encoding the peptide or the full-length protein, or by loading the cells with the peptides at issue following a peptide loading procedure as described by, e.g., Nestle et al. (*Nature Medicine* 4: 328-332, 1998).

For treatment purposes, the isolated CTL clones, the peptides or the peptide/HLA complexes, or the cells expressing the peptide/HLA complexes, can be administered to a subject alone or in combination with other appropriate materials, such as cytokines, adjuvants or a pharmaceutical carriers. The amount of the CTL cells, the peptides, the peptide/HLA complexes, or cells expressing the complexes, can be determined according the condition of the subject.

For additional teachings of diagnostic and therapeutic uses of isolated CTLs and peptide/HLA complexes, see, e.g., Thomson et al. (1995) *PNAS 92*: 5845; Altman et al. (1996) *Science 274*: 94-96; Dunbar et al. (1998) *Current Biology 8*: 413-416; Greenberg et al. (1986) *J. Immunol. 136*: 1917; and Kast et al. (1989) *Cell 59*: 603-614.

The present invention is further illustrated by the following examples.

All the publications mentioned in the present disclosure are incorporated herein by reference. The terms and expressions which have been employed in the present disclosure are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, recognizing that various modifications are possible within the scope of the invention.

EXAMPLE 1
GENERATION OF RECOMBINANT *YERSINIA* AND TARGETING EBV-TRANSFORMED B CELLS WITH RECOMBINANT *YERSINIA*

Strains, Plasmids and Growth Conditions

Y.enterocolitica strain E40(pYV40), MRS40(pYV40), which is the isogeneic ampicillin sensitive derivative of E40(pYV40), and their various non-polar mutants (Sory et al. (1995), *Proc. Natl Acad. Sci. USA 92*: 11998-12002). Plasmids are listed in **Table 3**. Bacteria were grown in Brain Heart Infusion (BHI) (Difco, Detroit, Michigan). After overnight preculture, bacteria were diluted 1/20 in fresh BHI, allowed to grow for 30 minutes at room temperature, and synthesis of the Yop virulon was induced by incubation for 150 minutes at 37°C before infection.

Construction of the Polymutant *Yersinia* Strains

To construct the *yopHOPEM* polymutant strain, the *yopE*, *yopH*, *yopO*, *yopM* and *yopP* genes were successively knocked out by allelic exchange in the MRS40 strain using the suicide vectors pMRS101 and pKNG101. See, K. Kaniga et al. (1991) "A wide-host range suicide vector for improving reverse genetics in gram-negative bacteria: inactivation of the blaA gene of *Yersinia enterocolitica*" *Gene 109*: 137-141 and M.R. Sarker et al. (1997) "An improved version of suicide vector pKNG101 for gene replacement in Gram-negative bacteria" *Mol. Microbiol. 23*: 409-411. The various deletions are described in Table 2 in the "suicide vectors and mutators" section. The *YopE* gene was first mutated using the mutator pPW52 (see, P. Wattiau et al. (1993) "SycE, a chaperone-like protein of *Yersinia enterocolitica*

involved in the secretion of YopE" *Mol. Microbiol. 8*: 123-131), giving strain MRS40(pAB4052). Mutation of the *YopH* gene in this strain with the mutator pAB31 (see, S.D. Mills et al. (1997) "*Yersinia enterocolitica* induces apoptosis in macrophages by a process requiring functional type III secretion and translocation mechanisms and involving YopP, presumably acting as an effector protein" *Proc. Natl. Acad. Sci. USA 94*: 12638-12643) gave the double *yopEH* mutant MRS40(pAB404). The triple *yopEHO* mutant MRS40(pAB405) was then obtained by allelic exchange with the mutator pAB34 (see, S.D. Mills et al., 1997). The *YopP* gene was then mutated with mutator pMSK7 (see S.D. Mills et al. (1997)), leading to the *yopEHOP* mutant MRS40(pMSK46). The *yopHOPEM* strain MRS40(pABL403) was finally obtained by allelic exchange with the *yopM* mutator pAB38 (see, S.D. Mills et al., 1997).

Table 3: Plasmids

Plasmids	Relevant Characteristics	References
pABL403	pYV40 $yopE_{21}$, $yopH\Delta^{1-352}$ $yopO\Delta^{65-558}$, $yopP_{23}$, $yopM_{23}$	see Example 2 of the present specification
	Suicide Vectors and mutators	
pKNG101	^{ori}R6K ^{sac}BR+ ^{on}TRK2 ^{str}AB+	K. Kaniga et al. (1991) *Gene 109*: 137-141.
pMRS101	^{ori}R6K ^{sac}BR+ ^{on}TRK2 ^{str}AB+ ^{ori}ColE1 *bla* +	M.R. Sarker and G.R. Cornelis (1997) *Mol. Microbiol. 23*: 409-411.

pAB31	pMRS101 yopH$_{\Delta 1-352}$+	S.D. Mills et al. (1997) *Proc. Natl. Acad. Sci. USA 94*: 12638-12643.
pAB34	pMRS101 yopO$_{\Delta 65-558}$+	S.D. Mills et al. (1997)
pAB38	pMRS101 yopM$_{23}$+	S.D. Mills et al. (1997)
pMSK7	pMRS101 yopP$_{23}$+	S.D. Mills et al. (1997)
pPW52	pKNG101 yopE$_{21}$+	P. Wattiau and G.R. Cornelis (1993) *Mol. Microbiol. 8*: 123-131.

Generation of recombinant *Yersinia* containing YopE$_{130}$-MAGE-A1

The sequence encoding protein MAGE-A1 was inserted in frame with a sequence encoding a truncated YopE, YopE$_{130}$, containing the first 130 amino acids of YopE. Such a plasmid is graphically depicted in **Figure 1**.

The open reading frame of MAGE-A1 was amplified by PCR using a MAGE-A1 cDNA cloned in pcDNAI/Amp (Invitrogen, Carlsbad, California) as template. The upstream primer, AAACTGCAGATGTCTCTTGAGCAGAGGAGTC (SEQ ID NO: 33), consisted of the first nucleotides of the open reading frame of MAGE-A1 preceded by a PstI site. The downstream primer, AAACTGCAGTCAGACTCCCTCTTCCTCCTC (SEQ ID NO: 34), consisted of nucleotides complementary to the last nucleotides of the open reading frame of MAGE-A1 followed by a PstI site. The PCR product was digested with PstI and inserted in frame with the truncated *YopE* at the PstI site of vector pMS111 (see, Sory et al. (1994) *Molecular Microbiology 14*: 583-594), to yield plasmid YopE$_{130}$-MAGE-A1 or pMS111-MAGE-A1.

pMS111-MAGE-A1 ($YopE_{130}$-MAGE-A1) was electroporated in bacteria strain DH5 F'IQ. DNA was extracted from some clones and the DNA of a positive recombinant clone was electroporated in bacteria strain SM10. After mobilization of pMS111 from SM10 in *Yersinia* MRS40 (pABL403), recombinant clones were then selected on agar-containing medium, supplemented with nalidixic acid, sodium-arsenite and chloramphenicol. MRS40 is an isogeneic derivative of E40 sensitive to ampicillin (see, Sory et al. (1995) *Proc. Natl. Acad. Sci. USA 92*: 11998-12002).

Generation of recombinant *Yersinia* containing $YopE_{130}$-MAGE-A4

The sequence encoding protein MAGE-A4 was linked in frame to a sequence encoding a truncated YopE, YopE (1-130), containing the first 130 amino acids of YopE. The open reading frame of MAGE-A4 was amplified by PCR using a MAGE-A4 cDNA cloned in pcDNAI/Amp (Invitrogen) as template. The upstream primer, AAAAACTGCAGATGTCTTCTGAGCAGAAGAGT (SEQ ID NO: 35), consisted of the first nucleotides of the open reading frame of MAGE-A4 preceded by a PstI site. The downstream primer, AAAAAATCGATTCAGACTCCCTCTTCCTC (SEQ ID NO: 36), consisted of nucleotides complementary to the last nucleotides of the open reading frame of MAGE-A4 followed by a ClaI site. The PCR was performed for 30 cycles (1 min at 94°C, 2 min at 55°C and 2 min at 72°C). The PCR product was digested with PstI and ClaI and inserted in frame with the truncated YopE at the PstI-ClaI sites of vector pMS621. Plasmid pMS621-MAGE-A4 was transformed into bacteria strain DH5αF'IQ by electroporation. Positive clones were detected by PCR on bacterial

colonies and the DNA of a positive recombinant clone was extracted and transformed into bacteria strain SM10 by electroporation. After mobilization of pMS621-MAGE-A4 from SM10 into polymutant *Yersinia* MRS40 (pABL403), recombinant clones were then selected on agar-containing medium, supplemented with nalidixic acid, sodium m-arsenite and chloramphenicol.

Generation of recombinant *Yersinia*-MAGE-A3

Yersinia-MAGE-A3(aa147-314)

The sequences encoding the truncated MAGE-A3 was amplified by PCR using a MAGE-A3 cDNA cloned in pcDNAI/Amp as template. The upstream primer PVB157 was 5'-AA **CTGCAG** TTTCCTGTGATCTTCAGCAAAGC-3'(SEQ ID NO: 50), consisting of nucleotides 439-461 of the open reading frame of MAGE-A3 (start codon ATG is 1-3), preceded by a PstI site (in bold) and 2 A's. The downstream primer PVB139, 5'-CC **ATCGAT** TCACTCTTCCCCCTCTCTCAA-3'(SEQ ID NO: 51), consisting of nucleotides complementary to the last nucleotides of the open reading frame of MAGE-A3 preceded by a ClaI site (in bold) and 2C's. PCR was performed for 30 cycles (1 min at 94°C, 2 min at 62°C and 2 min at 72°C). The PCR products were treated as described for MAGE-A4 hereinabove.

Construction of Yersinia-MAGE-A3(aa1-199)

To obtain the sequence encoding the first 199 amino-acids of the MAGE-A3 protein, the primers were designed as follows: upstream primer PVB172, 5'-ACCAGAGTCATC **CTGCAG** ATGCCTCTTGAG -3' (SEQ ID NO: 52), consisting of the nucleotides overlapping the start codon (underlined) of the open reading frame of MAGE-A3 preceded by a PstI site (in bold). The downstream primer

PVB 173, 5'-GCCTGCCTTGGG **ATCGAT** TCACATGATCTGATT-3'(SEQ ID NO: 53), consisting of nucleotides complementary to the nucleotides 577-600 of the open reading frame of MAGE-A3 and containing a ClaI site (in bold). PCR was performed for 30 cycles (1 min at 94°C, 2 min at 65°C and 2 min at 72°C). The PCR products were treated as described hereinabove for MAGE-A4.

Targeting EBV-Transformed B Cells with Recombinant *Yersinia*

Infection of EBV-transformed B cells with *Yersinia* MRS40 (pABL403) containing pMS111-MAGE-A1 was carried out as follows. Infection of EBV-transformed B cells with other recombinant *Yersinia* was carried out following essentiallly the same procedure.

One colony of *Yersinia* MRS40 (pABL403) containing pMS111-MAGE-A1 was then grown overnight at 28°C in LB medium supplemented with nalidixic acid (35 µg/ml), sodium m-arsenite (1 mM) and chloramphenicol (12 µg/ml). The overnight culture was diluted in fresh medium in order to obtain an OD (optical density) of 0.2 at 600nm after amplifying the fresh culture at 28°C for approximately 2 hours. The bacteria were washed in 0.9% NaCl and resuspended at 10^8 bacteria per ml in 0.9% NaCl assuming that a culture giving an OD_{600} equal to 1 contains 5 x 10^8 bacteria per ml. Irradiated EBV-B cells (100Gy) were resuspended at 10^6 in 3.8 ml of RPMI without antibiotics, supplemented with 10% FCS and AAG (L-Arginine (116 mg/ml), L-Asparagine (36 mg/ml) and L-Glutamine (216 mg/ml)). Then 200 µl of the bacterial suspension was added. Two hours after infection, gentamicin (30 µg/ml) was added for the next two hours,

and the cells were finally washed three times before being used as stimulator cells.

As a negative control, the same cells were also infected with *Yersinia* MRS40 (pABL403) containing pMS621, a plasmid which encodes only the truncated YopE, i.e., $YopE_{130}$.

EBV-B Cells infected with the recombinant *Yersinia*-MAGE-A1 were recognized by MZ2-CTL 82/30. MZ2-CTL 82/30 are specific for the MAGE-A1 peptide EADPTGHSY (SEQ ID NO: 1) which is presented by HLA-A1 (U.S. Patent No. 5,342,774). 5000 MZ2-CTL 82/30 cells were added in each microwell containing the *Yersinia* in a final volume of 100 μl of Iscove's complete medium (culture medium was supplemented with 10% human serum, L-arginine (116 mg/ml), L-asparagine (36 mg/ml), L-glutamine (216 mg/ml), streptomycine (0.1 mg/ml), penicillin (200 U/ml), IL-2 (25 U/ml) and gentamicin (15 μg/ml). After overnight incubation, the presence of IFN-gamma (that is produced by CTL upon activation) in the supernatant of the co-culture was tested in a standard ELISA assay (Biosource, Fleurus, Belgium). **Figure 2A** graphically depicts such a procedure.

As indicated in **Figure 2B**, the HLA-$A1^+$ B cells infected with *Yersinia* encoding $YopE_{130}$-MAGE-A1 were recognized by the CTL 82/30, while the same cells infected with the control plasmid $YopE_{130}$ were not. The optimal concentration of bacteria is around 1,000,000 per microwell.

EXAMPLE 2
GENERATION OF RECOMBINANT VACCINIA WR VIRUSES

Parental WR strain of Vaccinia (vP1170) contained the parent vector pKILGPT of 2826 bp (Virogenetics, Troy, New York). A sequence coding for MAGE-A1, placed after the Vaccinia Virus H6 promoter, was cloned into the pKILGPT vector, creating donor plasmid MAW035. A similar MAGE-A4 donor plasmid vector was constructed by replacing the MAGE-A1 cDNA with the MAGE-A4 cDNA. The MAGE-A3 cDNA was digested with Pst1 and XbaI, blunt ended, the insert was gel purified and ligated into the SmaI site of the PsC11 vector. For the PsC11 vector, see Chakrabati et al. (1985) *Mol. Cell Biol.* 5: 34-3-3409.

The donor plasmids was transfected into CEF cells containing the genomic DNA of vaccinia strain WR, yielding recombinant vaccinia viruses WR-MAGE-A1, WR-MAGE-A4 and WR-MAGE-A3, respectively, by way of *in vivo* recombination and selected with BrdU and X-gal. The procedure can be found in, e.g., Perkins et al. (1989) *J. Virol.* 63: 3829-3936.

EXAMPLE 3
GENERATION OF RECOMBINANT ALVAC-MAGE-A1 VIRUSES

A MAGE-A1 coding sequence, placed after the Vaccinia Virus H6 promoter, was cloned into the pUC8-based vector to generate donor plasmid MAW036.

Recombinant ALVAC-MAGE-A1 virus was generated by using the donor plasmid MAW036 and following well known procedures, e.g., as described in *Current Protocols in Molecular Cloning* (Ausubel et al., John Wiley & Sons, New York) and Ferrari et al. (*Blood* 90: 2406-2416, 1997).

Recombinant canarypox virus ALVAC-MAGE-A3 split (also referred to herein as "ALVAC-MAGE-A3" for simplicity) expressed two truncated overlapping fragments of MAGE-A3, one fragment spanning amino acids 1-196 and the other fragment spanning amino acids 147-199. In the recombinant virus, each fragment was contained in a separate expression cassette, each under the control of the vaccinia virus H6 promoter, and both cassettes were inserted at the C3 site in the ALVAC genome.

The vCP1563 recombinant was generated as follows. The MAGE-A3 (1-196) DNA fragment was generated and linked to the vaccinia H6 promoter by standard PCR procedures with plasmid pTZ18R (containing full length MAGE-A3 cDNA) as template. The MAGE-A3 (147-199) DNA fragment was generated and linked to the H6 promoter in the same way. These two fragments were then subcloned into a plasmid such that the cassettes were flanked by ALVAC DNA from the C3 insertion site. The organization of these elements in the plasmid was as follows: ALVAC C3 left flanking arm, MAGE-A3 (147-299)/H6, MAGE-A3 (1-196)/H6, ALVAC C3 right flanking arm. This ALVAC C3 site donor plasmid containing the MAGE-A3 (1-196) and (147-

299) fragment expression cassettes was designated pC3MAGE3 split.

The ALVAC-MAGE-A3 recombinant was generated by *in vivo* recombination between the pC3MAGE3 split donor plasmid and ALVAC genomic DNA following standard procedures. Recombinant virus was selected by hybridization with MAGE-3-specific DNA probes and plaque was purified. The resulting ALVAC-MAGE-A3 recombinant was given the laboratory designation vCP1563. Expression analysis with MAGE-3-specific antisera confirmed the expression of MAGE-A3 (1-196) and (147-299) polypeptides in cells infected with ALVAC-MAGE-A3 (vCP1563).

EXAMPLE 4
GENERATION OF RECOMBINANT ADENOVIRUSES

For the construction of the recombinant adenovirus, the plasmid pAd-CMVIcpA-MAGE-A4 (containing the MAGE-A4 cDNA under the control of the CMV promoter) was obtained by inserting into the NotI site of vector pAd-CMVIcpA (provided by Celia GARCIA and Thierry RAGOT, URA CNRS 1301), the MAGE-A4 complete cDNA.

The recombinant adenovirus Ad-MAGE-A4 was generated by *in vivo* homologous recombination in cell line 293 between pAd-CMVIcpA-MAGE-A4 and Ad-ßgal genomic DNA. Briefly, 293 cells were cotransfected with 5 μg of plasmid pAd-CMVIcpA-MAGE-A4 linearized with XmnI and 5 μg of the large ClaI fragment of Adeno-ßgal DNA (Stratford-Perricaudet et al. (1992), *J. Clin. Invest.*, 90: 626-630 and Patent FR 9603207. The recombinant adenovirus was plaque purified and the presence of the transgene was assessed by restriction analysis of the adenoviral DNA. Recombinant adenoviruses were propagated in 293 cells and purified by double cesium chloride density centrifugation. The viral stocks were stored in aliquots with 10% glycerol in liquid nitrogen and titered by plaque assay using 293 cells.

Recombinant adenovirus Ad-MAGE-A3 was generated according to essentially the same procedure described above, but MAGE-A3 cDNA was derived from a λgt10 recombinant clone.

EXAMPLE 5
RECOMBINANT RETROVIRUS
AND INFECTION OF CELL LINES

The M1-CSM retroviral vector encodes the full length MAGE-A1 protein, under the control of the LTR, and the truncated form of the human low affinity nerve growth factor receptor (ΔLNGFr) driven by the SV40 promoter (Mavilio F. et al., *Blood* 83: 1988-1997, 1994). EBV-B cells or PHA-activated T cells were transduced by coculture with irradiated packaging cell lines producing the M1-CSM vector in the presence of polybrene (8 µg/ml). After 72 hours, lymphocytes were harvested and seeded in fresh medium. The percentage of infected cells was evaluated 48 hours later by flow cytomemtry for LNGFr expression with the mAb 20.4 (ATCC, Rockville, MD, USA). The LNGFr positive cells were purified by magnetic cell sorting using Rat anti-mouse IgG1-coated beads (Dynabeads M-450, DYNAL A.S. N012 Oslo, Norway).

EXAMPLE 6
MATERIALS AND METHODS

Cell lines and Media

The Epstein Barr Virus (EBV) immortalized B cells (hereafter referred as to EBV-B cells) were obtained following the standard protocol. EBV-B cells and the melanoma cell lines were cultured in Iscove's modified Dulbecco medium (IMDM) (GIBCO BRL, Baitherburg, MD, USA) supplemented with 10% fetal calf serum (FCS) (GIBCO BRL), 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine (AAG), 100 U/ml penicillin and 100 µg/ml streptomycin. Hela and COS-7 cells were maintained in H16 medium (GIBCO BRL) supplemented with 10% FCS.

Cytokines

Human recombinant IL-2 was purchased from CHIRON BV (Amsterdam, Netherlands) or EUROCETUS (Amsterdam, Netherlands), or provided by BIOGEN (Geneva, Switzerland). Human recombinant IL-7 was purchased from GENZYME (Cambridge, MA). Human recombinant GM-CSF was purchased from SANDOZ (Leucomax, Sandoz Pharma, Basel, Switzerland) or SCHERING PLOUGH (Brinny, Ireland). Human recombinant IL-4, IL-6 and IL-12 were produced by the present inventors.

Processing of human blood

Peripheral blood was obtained from the local blood bank (non cancer patients, namely, hemochromatosis patients) as standard buffy coat preparations. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Lymphoprep (NYCOMED PHARMA, Oslo, Norway). In order to minimize contamination of PBMC by

platelets, the preparation was first centrifuged for 20 min at 1000 rpm at room temperature. After removal of the top 20-25 ml containing most of the platelets, the tubes were centrifuged for 20 min at 1500 rpm at room temperature. PBMC were depleted of T cells by rosetting with sheep erythocytes (BIO MÉRIEUX, Marcy-l'Etoile, France) treated with 2-aminoethyl-isothiouronium (SIGMA, St. Louis, MO, USA). Rosetted T cells were treated with NH_4Cl (160 mM) to lyse the sheep erythrocytes and washed. The $CD8^+$ T lymphocytes were isolated by positive selection using an anti-CD8 monoclonal antibody coupled to magnetic microbeads (MILTENYI BIOTECH, Germany) and by sorting through a magnet. The $CD8^+$ T lymphocytes were frozen, and thawed the day before the start of the primary culture and cultured overnight in Iscove's medium containing L-asparagine (0.24 mM), L-arginine (0.55 mM), L-glutamine (1.5 mM), 10% human serum (hereafter referred to as complete Iscove's medium) and supplemented with 2.5 U/ml IL-2.

The lymphocyte-depleted PBMC were frozen or used immediately for dendritic cell cultures. Cells were left to adhere for 1-2 hrs at 37°C in culture flasks (Falcon, BECTON DICKINSON LABWARE, Franklin Lakes, USA) at a density of 2×10^6 cells/ml in RPMI 1640 medium (GIBCO BRL) supplemented with L-asparagine (0.24 mM), L-arginine (0.55 mM), L-glutamine (1.5 mM) and 10% fetal calf serum (hereinafter referred to as complete RPMI medium). Non-adherent cells were discarded and adherent cells were cultured in the presence of IL-4 (100 U/ml) and GM-CSF (100 ng/ml) in complete RPMI medium. For experiments in Examples 7 and 8, cultures were fed on day 2 and day 4 by removing 1/3 of the volume of the medium and adding fresh medium with IL-4 (100 U/ml) and GM-CSF

(100 ng/ml); and on day 6 or 7, the non-adherent cell population was used as a source of enriched dendritic cells. For experiments in Example 9, cultures were fed on day 2 by removing 1/3 of the volume of the medium and adding fresh medium with IL-4 (100 U/ml) and GM-CSF (100 ng/ml) and were frozen on day 4; and on the day before each stimulation, dendritic cells were thawed and grown overnight in complete medium supplemented with 100 U/ml IL-4 and 100 ng/ml GM-CSF. For experiments in Examples 10-11, cultures were fed on day 2 and 4 by removing 1/2 or 1/3 of the volume of the medium and adding fresh medium with IL-4 (100 U/ml) and GM-CSF (100 ng/ml); and on day 5 or day 7, the non-adherent cell population was used as a source of enriched dendritic cells.

Interferon γ production assay.

5000 target cells were cultured overnight with 2000 CTL in 100 µl per well complete Iscove's medium supplemented with 25 U/ml IL-2 in 96 well round bottom plates. The production of interferon γ (IFN-γ) was measured in 50 µl supernatant by ELISA (Biosource).

cDNAs encoding HLA-class I molecules

The HLA-A*0201 coding sequence was obtained from a cDNA library of cell line BB49, cloned into expression vector pcDNAI/Amp (INVITROGEN). The HLA-A3 coding sequence was isolated from a cDNA library of cell line LB33 cloned into expression vector pcDNA3 (INVITROGEN). The HLA-B*4402 coding sequence was isolated by RT-PCR from cell line LB33 and cloned in expression vector pcDNAI/Amp. The HLA-B*40012 (B60) coding sequence was derived by RT-PCR from cell line HA7-RCC and cloned in expression vector pcDNA3. The HLA-Cw3

coding sequence was cloned in expression vector pCR3. The HLA-Cw5 was isolated from cell line LB373 by RT-PCR and cloned into pcDNA3. The HLA-B*0801, B*4002 (B61), Cw*02022, and Cw*0701 coding sequences were amplified by RT-PCR using RNA of LB 1118-EBV-B cells as the template. The HLA-B*5301 coding sequence was amplified by RT-PCR using RNA of EBV-B cells of patient LB 1118 as the template. The HLA-B7 and HLA-B40 segments were isolated as described in Examples 12 and 13. PCR products were cloned into expression vector pcDNA3. DNA was extracted from recombinant clones and sequenced partially on the sense and partially on the antisense strand by the dideoxy-chain termination method (Thermosequenase™ cycle sequencing kit, Amersham).

Peptides recognition assay

Peptides were synthesized on solid phase using F-moc for transient NH2-terminal protection and were characterized using mass spectrometry. All peptides were >80% pure, as indicated by analytical HPLC. Lyophilized peptides were dissolved in DMSO and stored at -20°C. Target cells were labeled with $Na(^{51}Cr)O_4$, washed, and incubated for 15 min in the presence of peptide. CTL clone was then added at an effector-to-target ratio of 5:1 to 10:1. Chromium release was measured after incubation at 37°C for 4 hours.

Example 7
A MAGE-A1 DERIVED PEPTIDE PRESENTED BY HLA-Cw3 MOLECULES TO CYTOLYTIC T LYMPHOCYTES

Isolation of MAGE-A1 specific CTL clone LB1137 462/F3.2

Autologous dendritic cells from donor LB 1137 (HLA-A2 A3 B4402 B60 Cw3 Cw5) were infected with the ALVAC-MAGE-A1 at a multiplicity of infection of 30 in RPMI containing 10% FCS at 37°C under 5% CO_2. After 2 hours, the infected dendritic cells were washed. For *in vitro* stimulation, 150,000 $CD8^+$ T lymphocytes and 30,000 infected dendritic cells were cocultured in microwells in 200 μl Iscove's medium containing L-asparagine (0.24 mM), L-arginine (0.55 mM), L-glutamine (1.5 mM), 10% human serum (hereafter referred to as complete Iscove's medium) and supplemented with IL-6 (1000 U/ml) and IL-12 (10 ng/ml). The $CD8^+$ lymphocytes were weekly restimulated with autologous dendritic cells freshly infected with the ALVAC-MAGE-A1 and grown in complete Iscove's medium supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml).

After several rounds of stimulation, an aliquot of each microculture was tested for specific lysis of autologous target cells. Autologous EBV-B cells were infected for two hours with either the parental vaccinia WR (batch LVAR) or the WR-MAGE-A1 construct (vP 1267), using a multiplicity of infection of 20, and labeled with $Na(^{51}Cr)O_4$. Afterwards, EBV-B cells (target cells) were washed, and added to the responder cells at an effector to target ratio of approximately 40:1. Unlabeled K562 cells were also added (5×10^4 per V-bottomed microwell) to block natural killer activity. Chromium release was measured after incubation at 37°C for 4 hours. The individual microcultures were tested in duplicate on each target.

The positive microcultures were cloned by limiting dilution, using autologous EBV-B cells infected with recombinant *Yersinia* expressing the $YopE_{130}$-MAGE-A1 protein as stimulating cells, and allogeneic EBV-B cells (LG2-EBV) as feeder cells. The cultures were restimulated similarly on day 7, and clones were maintained in culture by weekly restimulation with allogeneic EBV-B cells (LG2-EBV) in complete Iscove's medium supplemented with 0.5 µg/ml PHA-HA16 (Murex) and 50 U/ml of IL-2. At day 3 after restimulation, the clones were washed to remove the PHA-HA16 in the culture medium. The clones were tested for specific lysis of autologous EBV-B cells infected with the vaccinia-MAGE-A1 construct. Clone LB1137 462/F3.2 was found positive (**Figure 3a**) and used in subsequent experiments.

The MAGE-A1 epitope is presented to CTL by HLA-Cw3 molecules

As donor LB 1137 expresses a number of different HLA molecules as described supra, each HLA was tested to determine which one presented the antigen recognized by CTL LB1137 462/F3.2.

COS cells were transfected with plasmids encoding one of the six HLA-class I molecules together with the cDNA of MAGE-A1. In brief, 1.5 x 10^4 COS cells distributed in microwells were cotransfected with 50 ng of plasmid pcDNAI containing the MAGE-A1 cDNA and 50 ng of plasmid pcDNA3 containing the cDNA coding for one of the six HLA-class I molecules that were expressed by donor LB1137, using 1 µl of Lipofectamine reagent (Gibco BRL). The COS cells were incubated 5 hours at 37°C and 8% CO_2 in the transfection mixture and 200 µl of culture medium was added. After overnight culture, transfectants

were tested for their ability to stimulate the production of IFN-γ by clone LB1137 462/F3.2. Briefly, 1500 CTLs were added to each microwell containing transfected cells, in a final volume of 100 µl of Iscove's complete medium containing 25 U/ml of IL-2. After 24 hours, 50 µl supernatant was tested for its IFN-γ content in a WEHI bioassay which measured the cytotoxic effect of IFN-γ on cells of WEHI-164 clone 13 in a MTT colorimetric assay. Only those cells transfected with both HLA-Cw3 and MAGE-A1 stimulated CTL clone LB1137 462/F3.2 to produce IFN-γ (**Figure 3b**). COS cells transfected with MAGE-A1 or HLA-Cw3 alone did not stimulate the CTL clone.

Antigenic peptides and CTL assay.

In order to identify the MAGE-A1 peptide recognized by clone LB1137 462/F3.2, peptides (16 amino-acids) corresponding to parts of the MAGE-A1 protein were synthesized, loaded on the autologous EBV-B cells and tested for recognition. Peptides were synthesized on solid phase using F-moc for transient NH_2-terminal protection. Lyophilized peptides were dissolved at 20 mg/ml in DMSO, diluted at 2 mg/ml in 10 mM acetic acid and stored at -20°C.

Peptides were tested in chromium release assays in which 1000 ^{51}Cr-labeled target cells were incubated with 10 µg/ml of peptide in 96-well microplates (100 µl/well) for 20 min at room temperature, prior to adding 100 µl medium containing 10,000 CTL. The assay was terminated after 4 hours of incubation at 37°C and 8% CO2.

Autologous EBV-B cells incubated with peptide DGREHSAYGEPRKLLT (MAGE-A1 $_{225-240}$) (SEQ ID NO: 37) were recognized by CTL LB1137 462/F3.2 (Figure 3c). This long

peptide contained a 9-amino-acid peptide SAYGEPRKL (MAGE-A1 $_{230-238}$) (SEQ ID NO: 2) which contained adequate anchor residues for HLA-Cw3: a Y in position 3 and a L at the C-terminus. DGREHSAYGEPRKLLT (SEQ ID NO: 37) was screened for prediction of an HLA-Cw3 binding peptide with the software available at "http://bimas.dcrt.nih.gov/molbio/hla_bind /index.html". Peptide SAYGEPRKL (MAGE-A1 $_{230-238}$) (SEQ ID NO: 2) had the highest score for binding to HLA-Cw3. It was recognized by CTLLB1137 462/F3.2 in a cytotoxicity assay at an effector to target ratio of 10:1 (**Figure 3c**).

Recognition by CTL clone LB1137 462/F3.2 of HLA-Cw3 positive tumor cells expressing MAGE-A1

The activation of CTL LB1137 462/F3.2 by tumor cell lines that express HLA-Cw3 and MAGE-A1 was tested in an IFN-γ production assay. CTL clone LB1137 462/F3.2 recognized the HLA-Cw3 positive tumor cell line LB17-MEL which expresses MAGE-A1 (**Figure 3d**). The melanoma cell line Mi 665/2 E+ clone 2, that was transfected with a genomic fragment containing the open reading frame of MAGE-A1 (as described in U.S. Patent NO. 5,342,774), was also recognized by clone LB1137 462/F3.2, whereas the parental cell line Mi 665/2 was not recognized.

Example 8
A MAGE-A1 DERIVED PEPTIDE PRESENTED BY HLA-B5301 MOLECULES TO CYTOLYTIC T LYMPHOCYTES

Isolation of MAGE-A1 specific CTL clone LB1801 456/H7.11

Autologous dendritic cells from donor LB1801 (HLA-A201, A28, B4401, B5301, Cw04, Cw0501) were infected with the ALVAC-MAGE-A1 construct at a multiplicity of infection of 30 in RPMI containing 10% FCS at 37°C under 5% CO_2. After 2 hours, the infected dendritic cells were washed twice. For *in vitro* stimulation, 150,000 CD8+ lymphocytes and 30,000 infected dendritic cells were cocultured in round bottomed microwells in 200 microliters Iscove's medium containing L-asparagine (0.24 mM), L-arginine (0.55 mM), L-glutamine (1.5 mM), 10% human serum (hereafter referred as complete Iscove's medium) and supplemented with IL-6 (1000 U/ml) and IL12 (10 ng/ml). The CD8+ lymphocytes were weekly restimulated with autologous dendritic cells freshly infected with the ALVAC-MAGE-A1 construct and grown in complete Iscove's medium supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml).

Autologous EBV-B cells were infected for 2 hours with either the parental vaccinia WR (vP1170) or the recombinant vaccinia WR-MAGE-A1 (vP1188) using a multiplicity of infection of 20, and labeled with $Na(^{51}Cr)O_4$. Target cells were washed, and added to the responder cells at an effector to target ratio of approximately 40:1. Unlabeled K562 were also added (5×10^4 per V-bottomed microwell) to block natural killer activity. Chromium release was measured after incubation at 37°C for 4 hours. The individual microcultures were tested in duplicate on each target.

The microcultures containing cells that specifically lysed autologous EBV-B cells infected with the vaccinia-MAGE-A1 construct were cloned by limiting dilution using autologous EBV-B cells previously infected with the *Yersinia* expressing $YopE_{1-130}$-MAGE-A1 as stimulating cells, and allogeneic EBV-B cells (LG2-EBV) as feeder cells. CTL clones were maintained in culture by weekly restimulation in complete Iscove's medium supplemented with 50 U/ml of IL2. The clones were tested for specific lysis of autologous EBV-B cells infected with the vaccinia-MAGE-A1 construct. Clone LB1801 456/H7.11 was found positive (Figure 4a) and used in the following experiments. The CTL was restimulated weekly with LG2-EBV as feeder cells and alternately, purified phytohaemagglutin (PHA-HA16; MUREX) (0.5 mg/ml) or autologous EBV-B cells previously infected with the *Yersinia*-$YopE_{1-130}$-MAGE-A1.

Antigenic peptides and CTL assay

In order to identify the MAGE-A1 peptide recognized by clone LB1801 456/H7.11, peptides (16 amino-acids) corresponding to parts of the MAGE-A1 protein were synthesized, loaded on the autologous EBV-B cells and tested for recognition. Peptides were synthesized on solid phase using F-moc for transient NH2-terminal protection. Lyophilized peptides were dissolved at 20 mg/ml in DMSO, diluted at 2 mg/ml in 10 mM acetic acid and stored at -20°C. Peptides were tested in chromium release assay where 1000 ^{51}Cr-labeled target cells were incubated for 15 min at room temperature in V-bottomed microplates with 5 μg/ml of peptide, before adding an equal volume containing 5,000 CTLs. The assay was terminated after 4 hours of incubation at 37°C and 8%

CO2. Peptides QVPDSDPARYEFLWGP (MAGE-A1 253-268) (SEQ ID NO: 38) and SDPARYEFLWGPRALA (MAGE-A1 257-272) (SEQ ID NO: 39) scored positive.

Identification of the HLA presenting molecule

To know which HLA molecule presented both 16-mers peptides to CTL clone LB1801 456/H7.11, peptides were tested in a chromium release assay using, as target cells, EBV-B cells from different donors that shared HLA molecules with donor LB1801. Clone LB1801 456/H7.11 were able to recognize the peptide only when presented by autologous cells (**Table 4**). Because, no EBV-B cells expressing the HLA-B5301 molecule was tested, the cDNA coding for HLA-B5301 of donor LB1801 was isolated.

The HLA-B5301 coding sequence was amplified by RT-PCR using RNA of LB1801-EBV-transformed B cells as template. The PCR products were cloned into expression vector pcDNA3 (Invitrogen BV, the Netherlands). DNA was extracted from recombinant clones and sequenced partially on the sense and partially on the antisense strand to check that it was a sequence encoding HLA-B5301. The sequence for HLA-B5301 is described by Mason and Pasham (1998), *Tissue Antigens* 51: 417-466.

COS-7 cells were transfected with plasmids encoding HLA-B5301 molecule together with MAGE-A1 cDNA. In brief, 1.5 x 10^4 COS-7 cells distributed in microwells were cotransfected with 100 ng of plasmid pcDNAI containing the MAGE-A1 cDNA, 100 ng of plasmid pcDNA3 containing the cDNA coding for HLA-B5301 molecule of donor LB 1801, and one microliter of lipofectamine (Gibco BRL). The COS-7 cells
were incubated 24 hours at 37°C and 8% CO_2. These transfectants were then tested for their ability to

stimulate the production of TNF by clone LB1801 456/H7.11. Briefly, 1,500 CTLs were added to the microwells containing

Table 4

Target Cells	HLA Typing			% of Lysis	
				No Peptide	SDPARYEFLWGPRALA
LB1801	A2 A28	B4402 B53	CwD4 Cw0501	5	41
LB1118	A2 A3	B8 B61	Cw2 Cw7	19	15
LB33	A24 A28	B13 B4402	Cw6 Cw7	22	18
LB1158	A2 A3	B35 B51	Cw1 Cw4	6	4
LB1137	A2 A3	B4402 B60	Cw3 Cw5	5	3
LG2	A24 A32	B3503 B4403	Cw4	1	4
LB1819	A2	B44 B57	Cw5 Cw7	0	4
LB1161	A3 A26	B39 B4402		1	8
LB1213	A24	B18 B35	Cw4 Cw7	0	4

Table 4: Lysis by CTL LB1801 456/H7.11 of various EBV-B cells (target cells) pulsed with MAGE-A1 peptide.

EBV-B cells were 51Cr labeled and incubated with CTL at an effector to target cell ratio of 5/1 in the presence (or not) of 5 microgrammes of peptide SDPARYEFLWGPRALA (SEQ ID NO: 39). Chromium release was measured after 4 hours.

the transfectants, in a total volume of 100 ml of Iscove's complete medium containing 25 U/ml of IL-2. After 24 hours, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on cells of WEHI-164 clone 13 in a standard MTT

colorimetric assay. The cells transfected with both HLA-B53 and MAGE-A1 stimulated CTL clone LB1801 456/H7.11 to produce TNF (**Figure 4b**). COS-7 cells transfected with MAGE-A1 or HLA-B53 alone did not stimulate the CTL clone.

Identification of the antigenic peptide

To identify the sequence of the shortest synthetic peptide recognized by clone LB1801 456/H7.11, we compared the lysis by the CTL of autologous EBV-B cells, loaded with the MAGE-A1 peptide SDPARYEFLWGPRALA (MAGE-A1 257-272) (SEQ ID NO: 39) or the MAGE-A4 peptide GSNPARYEFLWGPRAL (MAGE-A4 264-279) (SEQ ID NO: 40), in a chromium release at an effector target ratio of 10 and a final concentration of peptide of 5 μg/ml. The MAGE-A1 peptide, but not the MAGE-A4 peptide, was recognized. The 10-mer peptide SDPARYEFLW (SEQ ID NO: 41) and the 9-mer peptide DPARYEFLW (SEQ ID NO: 42) were then synthesized and tested in a cytotoxic assay at an effector to target ratio of 5. Both peptides were recognized. The shorter peptide was then tested at different concentration at an effector to target ratio of 10 (**Figure 4c**). Half-maximal lysis was obtained at between 10 and 100 ng/ml.

Example 9
A MAGE-A4 DERIVED PEPTIDE PRESENTED BY HLA-A2 MOLECULES TO CYTOLYTIC T LYMPHOCYTES

Isolation of MAGE-A4 specific CTL clone LB1137 H4.13

Autologous dendritic cells from donor LB 1137 (HLA-A2, -A3, -B4402, -B60, -Cw3, -Cw5) were infected with the Ad-MAGE-A4 construct at a multiplicity of infection of 200 in RPMI containing 10% FCS at 37°C under 5% CO_2. After 2 hours, the infected dendritic cells were washed. For *in vitro* stimulation, 150,000 $CD8^+$ T lymphocytes and 30,000 infected dendritic cells were cocultured in microwells in 200 µl Iscove's medium containing L-asparagine (0.24 mM), L-arginine (0.55 mM), L-glutamine (1.5 mM), 10% human serum (hereafter referred to as complete Iscove's medium) and supplemented with IL-6 (1000 U/ml) and IL-12 (10 ng/ml). The $CD8^+$ lymphocytes were weekly restimulated with autologous dendritic cells freshly infected with the Ad-MAGE-A4 construct and grown in complete Iscove's medium supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml) These cells were tested as responder cells in the following assay.

Autologous EBV-B cells were infected for 2 hours with either the parental vaccinia WR parent (batch vP1170 or batch L VAR) or the recombinant vaccinia WR-MAGE-A4 (batch vP1545) using a multiplicity of infection of 20, and labeled with $Na(^{51}Cr)O_4$. These target cells were washed, and added to the responder cells at an effector to target ratio of approximately 40:1. Unlabeled K562 cells were also added (5 x 10^4 per V-bottomed microwell) to block natural killer activity. Chromium release was measured after incubation at 37°C for 4 hours. The individual microcultures were tested in duplicate on each target.

The microcultures containing cells that specifically lysed autologous EBV-B cells infected with the vaccinia-MAGE-A4 construct were cloned by limiting dilution using, as stimulating cells, autologous EBV-B cells infected with the recombinant *Yersinia* expressing $YopE_{1-130}$-MAGE-A4 (described above), and using allogeneic EBV-B cells (LG2-EBV) as feeder cells. Infection of EBV-B cells with *Yersinia* $YopE_{1-130}$-MAGE-A4 was done as follows: one colony of Yersinia MRS40 (pABL403) containing pMS621-MAGE-A4 ($YopE_{1-130}$-MAGE-A4) was grown overnight at 28°C in LB medium supplemented with nalidixic acid, sodium m-arsenite and chloramphenicol. From this culture, a fresh culture at an OD (600 nm) of 0.2 was then amplified at 28°C for approximately 2 hours. The bacteria were then washed in 0.9 % NaCl and resuspended at 10^8 bacteria per ml in 0.9 % NaCl. Irradiated EBV-B cells were infected at a multiplicity of infection of 20 in complete RPMI 1640 (culture media was supplemented with 10% FCS, and with L-arginine (116 mg/ml), L-asparagine (36 mg/ml), L-glutamine (216 mg/ml). Two hours after infection, gentamycin (30 μg/ml) was added for the next two hours, and the cells were finally washed 3 times. CTL clones were maintained in culture by weekly restimulation with either *Yersinia* $YopE_{1-130}$-MAGE-A4 infected EBV-B cells, HLA-A2 melanoma cell line QUAR (LB1751-MEL) that expressed MAGE-A4, or PHA (0.5 μg/ml) in complete Iscove's medium supplemented with 50 U/ml of IL-2. The clones were tested for specific lysis of autologous EBV-B cells infected with the vaccinia-MAGE-A4 construct. Clone LB1137 H4.13 was found positive (**Figure 5a**) and used in the following experiments.

The MAGE-A4 epitope is presented to CTL by HLA-A2 molecules

The lysis by CTL clone LB1137 H4.13 of EBV-B cells infected with the vaccinia-MAGE-A4 construct was inhibited by addition of an anti-HLA-A2 monoclonal antibody but not by addition of an anti-HLA-A3 or an anti-HLA-B,C monoclonal antibody. This indicated that the MAGE-A4 epitope was presented by HLA-A2 molecules.

COS cells were transfected with plasmids encoding the HLA-A2 molecule together with the cDNA of MAGE-A4. In brief, 1.5 x 10^4 COS cells distributed in microwells were cotransfected with 50 ng of plasmid pcDNAI containing the MAGE-A4 cDNA, 50 ng of plasmid pcDNA1/Amp containing the genomic DNA coding for the HLA-A2 molecule and 1 µl of DMRIEC (Gibco BRL). The COS cells were incubated 24 hours at 37°C and 8% CO_2. These transfectants were then tested for their ability to stimulate the production of TNF by clone LB1137 H4.13. Briefly, 2000 CTL were added to the microwells containing the transfectants, in a total volume of 100 µl of Iscove's complete medium containing 25 U/ml of IL-2. After 24 hours, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on cells of WEHI-164 clone 13 in a standard MTT colorimetric assay. The cells transfected with both HLA-A2 and MAGE-A4 stimulated CTL clone LB1137 H4.13 to produce TNF (**Figure 5b**). COS cells transfected with MAGE-A4 or HLA-A2 alone did not stimulate the CTL clone.

Determination of the antigenic peptide

In order to identify the MAGE-A4 peptide recognized by clone LB1137 H4.13, PCR reactions were performed using the MAGE-A4 cDNA as template, an upstream

primer (S) consisting of the first nucleotides of the open reading frame of MAGE-A4 and 8 downstream primers (AS1 to AS8) (**Figure 6**), separated from each other by approximately 100-120 bp in the open reading frame of MAGE-A4. The PCR was performed for 30 cycles (1 min at 94°C, 2 min at 63°C and 3 min at 72°C. This led to the amplification of 8 fragments of MAGE-A4 of different lengths (MAGE-A4(1) to MAGE-A4(8)), the longer one (MAGE-A4(1)) containing the entire open reading frame of MAGE-A4. PCR products were ligated into the pcDNA3.1/V5/His-TOPO vector and the recombinant vectors were transformed into *E. coli* cells (Topo TA cloning kit, Invitrogen). Colonies were analyzed by PCR and DNA of positive clones was extracted and used to transfect HeLa cells together with a plasmid encoding the HLA-A2 molecule. Briefly, 2 x 10^4 HeLa cells distributed in microwells were cotransfected with 50 ng of plasmid pcDNA3.1/V5/His-TOPO containing the MAGE-4A fragment, 50 ng of plasmid pcDNA1/Amp containing the genomic DNA coding for the HLA-A2 molecule and 1 µl of Lipofectamine (Gibco BRL). The HeLa cells were incubated 24 hours at 37°C and 8% CO_2. These transfectants were then tested for their ability to stimulate the production of TNF by clone LB1137 H4.13 as described above. Transfection with inserts S-AS1 and S-AS2 were positive, transfections with the other constructs were negative. This led to the identification of a MAGE-4A fragment of 130 bp,

TGATGGGAGGGAGCACACTGTCTATGGGGAGCCCAGGAAACTGCTCACCCAAGATTG
GGTGCAGGAAAACTACCTGGAGTACCGGCAGGTACCCGGCAGTAATCCTGCGCGCTA
TGAGTTCCTGTGGGGT (SEQ ID NO: 43),

encoding the epitope recognized by clone LB1137 H4.13.

The sequence of the putative fragment of the MAGE-A4 protein encoded by this region was screened for

prediction of an HLA-A2 binding peptide with the software available at http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html". Peptide GVYDGREHTV(SEQ ID NO: 44) (MAGE-A4 $_{230-239}$) had the highest score. It was synthesized and tested in a cytotoxicity assay at an effector to target ratio of 10:1. Peptide GVYDGREHTV (MAGE-A4 $_{230-239}$)(SEQ ID NO: 44) was found to sensitize autologous target cells to lysis by clone LB1137 H4.13 (**Figure 5c**).

Recognition by CTL clone LB1137 H4.13 of HLA-A2 cells expressing MAGE-A4

As indicated in **Figure 5d**, CTL clone LB1137 H4.13 was able to lyse HLA-A2 melanoma cell line QUAR (LB1751-MEL) that expressed MAGE-A4.

EXAMPLE 10
A MAGE-A1 PEPTIDE PRESENTED BY HLA-Cw2 TO CTL CLONE LB1118 466/D3.31

Isolation Of CTL Clone LB1118 466/D3.31

Dendritic cells ($3x10^6$/ml) from donor LB 1118 (HLA-A*0201, A3, B*0801, B*4002, Cw*02022, Cw*0701) were infected with the ALVAC-MAGE-A1 at a multiplicity of infection of 30 in RPMI supplemented with AAG and 10% FCS at 37°C under 5% CO_2. After 2 hours, the infected dendritic cells were washed. 150,000 autologous $CD8^+$ T lymphocytes and 30,000 infected dendritic cells were cocultured in microwells in 200 μl complete Iscove's medium and supplemented with IL-6 (1000 U/ml) and IL-12 (10 ng/ml). The $CD8^+$ lymphocytes were restimulated on days 7 and 14 with autologous dendritic cells freshly infected with the ALVAC-MAGE-A1 and grown in complete Iscove's medium supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml).

The microcultures containing proliferating CD8+ T cells were assessed on day 21 for their capacity to lyse autologous EBV-B cells infected with vaccinia-MAGE-A1 (vP 1188). EBV-B cells infected with parental vaccinia (vP1170) were used as a negative control. Infected EBV-B cells (target cells) were washed and added to the responder cells at an effector to target ratio of approximately 40:1. Unlabeled K562 cells were also added (5×10^4 per V-bottomed microwell) to block natural killer activity. Chromium release was measured after incubation at 37°C for 4 hours. The individual microcultures were tested in duplicate on each target. In a first experiment, an anti-MAGE-A1 reactivity was detected in 3 microcultures out of 96. 13% of the microcultures contained responder cells that lysed targets infected

with either vaccinia or vaccinia-MAGE-A1, but not the uninfected targets. This result indicated that the ALVAC and vaccinia vectors shared antigens recognized by CTL. In a second experiment, 2 microcultures scored positive in their anti-MAGE-A1 reactivity.

The positive microcultures (i.e., those that recognize autologous EBV-B cells infected with vaccinia-MAGE-A1 construct) were cloned by limiting dilution using, as stimulating cells, either autologous PHA-activated T cells transduced with a retrovirus encoding MAGE-A1, or autologous EBV-B cells transduced with the same retrovirus ($5x10^3$ to 10^4 cells per well in a 96-well plate). Allogeneic EBV-B cells ($5x10^3$ to 10^4 LG2-EBV-B cells per well in a 96-well plate) were used as feeder cells. CTL clones were tested for specific lysis of autologous EBV-B cells infected with the vaccinia-MAGE-A1 construct. The established CTL clones were maintained in complete IMDM supplemented with IL-2 (50 U/ml) and 0.5 μg/ml purified PHA (instead of stimulator cells) and passaged by weekly restimulation with allogeneic EBV-B cells ($1.5x10^6$ LG2-EBV-B cells per well in a 24-well plate).

Clone LB1118 466/D3.31 was identified as a positive clone that recognized autologous EBV-B cells infected with vaccinia-MAGE-A1 (**Figure 7A**), or EBV-B transduced with a retrovirus encoding MAGE-A1.

Identification of the Peptide and the Presenting Molecule

To identify the HLA molecule that presents the MAGE-A1 peptide by CTL clone LB1118 466/D3.31, COS cells were transfected with the MAGE-A1 cDNA, together with cDNAs coding for one of the putative HLA presenting molecules. These transfected cells were then tested for

their ability to stimulate the CTL clone to produce TNF. CTL clone LB1118 466/D3.31 produced TNF upon stimulation by COS cells transfected with MAGE-A1 and HLA-Cw2 (**Figure 7B**). To identify the MAGE-A1 peptide recognized by this CTL clone, a set of MAGE-A1 peptides of 12 amino acids that overlapped by 8 amino acids were screened. Autologous EBV-B cells were incubated with each of these peptides at a concentration of 1 µM, and tested for recognition by the CTL in a chromium release assay. Peptide ASAFPTTINFTR (MAGE-$A1_{61-72}$)(SEQ ID NO: 45) scored positive whereas the 16 amino-acid peptide SPQGASAFPTTINFTR (MAGE-$A1_{57-72}$), (SEQ ID NO: 46) scored negative. As information was not available for the residues anchoring a peptide in an HLA-Cw2 molecules, a number of shorter peptides were tested. Peptide SAFPTTINF (MAGE-$A1_{62-70}$) (SEQ ID NO: 47) was subsequently found to be the shortest peptide capable of efficiently sensitizing autologous target cells to lysis by CTL clone LB1118 466/D3.31, with a half-maximal lysis obtained at ~0.1nM (**Figure 7C**). The natural processing of the antigen was shown by the lysis by CTL clone LB1118 466/D3.31 of HLA-Cw2 tumor cell lines that express MAGE-A1 (**Figure 7D**).

EXAMPLE 11
A MAGE-A1 PEPTIDE PRESENTED BY HLA-A28 TO CTL CLONE LB1801 456/H8.33

Dendritic cells were derived from donor LB 1801 (HLA-A*0201, A28, B*4401, B*5301, Cw4, Cw*0501). CTL clone LB1801 456/H8.33 was isolated by following essentially the same procedure as described in Example 10.

Briefly, immature dendritic cells derived from blood monocytes were infected with ALVAC-MAGE-A1 and used to stimulate autologous CD8+T cells in the presence of IL-6 and IL-12. Responder cells were restimulated once a week with autologous dendritic cells, infected with ALVAC-MAGE-A1, in the presence of IL-2 and IL-7. Responder cells were tested on day 28 for their lytic activity on autologous EBV-transformed B (EBV-B) cells infected with a vaccinia virus encoding MAGE-A1 (vaccinia-MAGE-A1).

Positive microcultures were subject to limiting dilution using EBV-B cells infected with *Yersinia*-MAGE-A1 as stimulating cells. CTL clone LB1801 456/H8.33 lysed autologous EBV-B cells infected with vaccinia-MAGE-A1 (**Figure 8A**). CTL clone LB1801 456/H8.33 produced TNF upon stimulation by COS-7 cells transfected with HLA-A28 and MAGE-A1 (**Figure 8B**). Peptide EVYDGREHSA (MAGE-A1$_{222-231}$)(SEQ ID NO: 48) produced half-maximal lysis of target cells at ~0.3 nM (**Figure 8C**). A tumor cell line expressing MAGE-A1 and HLA-A28 was lysed by the CTL, but the lysis was lower than that obtained with cells infected with vaccinia-MAGE-A1 (**Figure 8C**).

EXAMPLE 12
A MAGE-A3 PEPTIDE PRESENTED BY HLA-B40 TO CTLS

Processing of human blood

Blood samples from donor LB 1841 (HLA-A3, -B35, -B40, Cw3, -Cw4) were processed as described in Example 6, except that:

1. the interphase containing the PBMC was harvested and then washed 3 times (or more) in cold phosphate buffer solution with 2 mM EDTA in order to eliminate the remaining platelets;

2. monocyte-derived dendritic cells (DC) were frozen on day 6. On the day of stimulation, DC were thawed and infected with a recombinant adenovirus encoding MAGE-A3, at a multiplicity of infection (MOI) of 500.

Isolation of CTL clones specific for MAGE-A3

Dendritic cells obtained from the blood sample of donor LB 1841 were infected with ALVAC-MAGE-A3 and cocultured with autologous CD8+ cells following essentially the same procedure as described in Example 7.

After several rounds of stimulation, the microculture was tested for specific lysis of autologous target cells following essentially the same procedure as described in Example 7, except that the vaccinia samples were sonicated for 30 seconds prior to use for infection of EBV-B target cells.

Microculture 526/F7, identified as containing cells that specifically lysed autologous EBV-B cells infected with vaccinia-MAGE-A3, was cloned by limiting dilution using, as stimulating cells, autologous EBV-B cells infected with recombinant ALVAC-MAGE-A3 (vCP1563)

at a multiplicity of infection of 30 (the ALVAC-MAGE-A3 sample was also sonicated 30 sec before use), or with PHA, according to the following scheme:

First week: stimulators = ALVAC-MAGE-A3 (vCP1563) infected EBV-B cells in complete IMDM medium supplemented with 50 U IL-2/ml, 2.5 ng IL-12/ml and 5 U IL-4/ml;

Second week: stimulators = ALVAC-MAGE-A3 (vCP1563) infected EBV-B cells in complete IMDM medium supplemented with 50 U IL-2/ml;

Third week: PHA (0.5 µg/ml) in complete IMDM medium supplemented with 50 U/ml of IL-2;

Fourth week: PHA (0.5 µg/ml) in complete IMDM medium supplemented with 50 U/ml of IL-2, 5ng/ml of IL-7 + gentamicin (15 µg/ml) + MRA 0.5 g/ml (from ICN);

Fifth and sixth week: stimulators = EBV-B cells infected with Yersinia-MAGE-A3(aa147-314) and EBV-B cells infected with Yersinia-MAGE-A3(aa1-199) in complete IMDM medium supplemented with 50 U/ml of IL-2, 2.5ng/ml of IL-12, 5 U/ml Il-4 + gentamicin (15 µg/ml);

Seventh week and each week thereafter: PHA (0.2 µg/ml) in complete IMDM medium supplemented with 50 U/ml of IL-2, 5 U/ml of IL-4 + gentamicin (15 µg/ml).

CTL clones were maintained in culture by weekly restimulation.

The clones were tested for specific lysis of autologous EBV-immortalized B cells infected with the vaccinia-MAGE-A3 construct, or with the parental vaccinia as a negative control. Clone LB1841 526/F7.1 was found positive (**Figure 9A**) and was used in the following experiments.

The MAGE-A3 epitope is presented to CTL LB1841 526/F7.1 by HLA-B40.

To identify the HLA molecule that presents the MAGE-A3 epitope recognized by CTL clone LB1841 526/F7.1, COS cells were transfected with the MAGE-A3 cDNA together with cDNAs coding for each of the putative HLA presenting molecules. In brief, 1.5 x 10^4 COS cells distributed in microwells were cotransfected with 50 ng of plasmid pcDNA3-MAGE-A3, 50 ng of plasmid containing coding sequences for HLA molecules and 1 µl of Lipofectamine (Gibco BRL). The HLA coding sequences were isolated from various individuals; in particular, the HLA-B40 cDNA was obtained by RT-PCR using RNA from tumor cell line HA-7-RCC as a template. This PCR product cDNA was inserted in pcDNA3 (InVitrogen). The COS cells were incubated 24 hours at 37°C and 8% CO_2. These transfectants were then tested for their ability to stimulate the production of TNF by clone LB1841 526/F7.1. Briefly, 2500 CTLs were added to the microwells containing the transfectants, in a total volume of 100 µl of Iscove's complete medium containing 25 U/ml of IL-2. After 24 hours, the supernatant was collected and the TNF content was determined by testing the cytotoxic effect of the supernatant on cells of WEHI-164 clone 13 in a standard MTT colorimetric assay. The cells transfected with both HLA-B40 and MAGE-A3 stimulated CTL clone LB1841 526/F7.1 to produce TNF (**Figure 9B**). Cos cells transfected with MAGE-A3 or HLA-B40 alone did not stimulate the CTL clone.

Identification of the antigenic peptide

To identify the MAGE-A3 peptide recognized by CTL clone LB1841 526/F7.1, a set of peptides of 16 amino acids that overlapped by 12 amino acids and covered the

entire MAGE-A3 protein sequence, was screened. Autologous EBV-B cells were incubated with each of these peptides at a concentration of 1 µg/ml, and tested for recognition by CTL clone LB1841 526/F7.1 in a chromium release assay at an effector to target cell ratio of 5:1. Peptide AALSRKVAELVHFLLL (SEQ ID NO: 54) scored positive. The sequence of this peptide was screened for prediction of an HLA-B40 binding peptide with the software available at "http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html". Peptide AELVHFLLL (MAGE-A3 114-122) (SEQ ID NO: 55) had the highest score. It was tested in a cytotoxicity assay with CTL clone LB1841 526/F7.1 and produced half-maximal lysis of autologous EBV-B target cells at ~7nM (**Figure 9C**).

Recognition by CTL clone LB1841 526/F7.1 of HLA-B40 cells expressing MAGE-A3

CTL clone LB1841 526/F7.1 was also able to lyse melanoma cell lines that expresses MAGE-A3 which were obtained from HLA-B40 positive patients, e.g., LB-43-MEL (**Figure 9D**). Some melanoma cells, e.g., SK-MEL-28 and SK-31-MEL, were not lysed by clone LB1841 526/F7.1, even though the level of expression of MAGE-A3 appeared to be appropriate in these cells. The peptide-pulsed cells were also insensitive to the CTL lysis. The lack of lysis was likely due to downregulation of the HLA expression in these melanoma cells, since treatment of SK-MEL-28 cells with IFN-γ increased the lysis of these cells by clone LB1841 526/F7.1 (IFN-γ is known to upregulate HLA expression in certain cells).

EXAMPLE 13
A MAGE-A1 PEPTIDE PRESENTED BY HLA-B7 MOLECULES TO CYTOLYTIC T LYMPHOCYTES

Processing of human blood

Blood samples from donor LB 1803 (HLA-A2, -A32, B7, -B60) were processed as described in Example 12.

Isolation of CTL clones specific for MAGE-A1

Dendritic cells obtained from the blood sample of donor LB 1803 were infected with ALVAC-MAGE-A1 and cocultured with autologous CD8+ cells following essentially the same procedure as described in Example 7 except that 15µg/ml gentamycin was added to the culture medium.

After several rounds of stimulation, the microculture was tested for specific lysis of autologous target cells following essentially the same procedure as described in Example 7, except that the vaccinia samples were sonicated for 30 seconds prior to use for infection of EBV-B target cells.

Microculture 483/G8, identified as containing cells that specifically lysed autologous EBV-B cells infected with recombinant vaccinia (WR-MAGE-A1), was cloned by limiting dilution using, for the first two weekly stimulations, irradiated autologous EBV-B cells infected with Yersinia-MAGE-A1 as stimulating cells, irradiated LG2-EBV as feeder cells, IL-2 (50 U/ml) and gentamycin (15 µg/ml). CTL clones were then maintained in culture by weekly restimulation with PHA (0.5 µg/ml), feeder cells and IL-2.

The clones were tested for specific lysis of autologous EBV-immortalized B cells infected with recombinant vaccinia (WR-MAGE-A1), or with the parental

vaccinia as a negative control. Clone LB1803 483/G8.4 was found positive (**Figure 10A**) and was used in the following experiments.

The MAGE-A1 epitope is presented to CTL by HLA-B7 molecules.

Donor LB 1803 was typed HLA-A2, -A32, -B7, -B60. To identify the HLA molecule that presents the MAGE-A1 epitope recognized by CTL clone LB1803 483/G8.4, COS-7 cells were transfected with the MAGE-A1 cDNA together with cDNAs coding for the putative HLA presenting molecules. In brief, 1.5 x 10^4 COS-7 cells distributed in microwells were cotransfected with 50 ng of plasmid pcDNA3-MAGE-A1, 50 ng of plasmid containing coding sequences for HLA molecules and 1 µl of Lipofectamine (Gibco BRL). The HLA coding sequences were isolated from various individuals; in particular, the HLA-B7 cDNA was obtained by RT-PCR using RNA from tumor cell line LB23-SAR as a template. This PCR product was inserted in pcDSRalpha. The transfected COS-7 cells were incubated 24 hours at 37°C and 8% CO^2. These transfectants were then tested for their ability to stimulate the production of TNF by clone LB1803 483/G8.4. Briefly, 2000 CTL were added to the microwells containing the transfectants, in a total volume of 100 µl of Iscove's complete medium containing 25 U/ml of IL-2. After 24 hours, the supernatant was collected and the TNF content was determined by testing the cytotoxic effect of the supernatant on cells of WEHI-164 clone 13 in a standard MTT colorimetric assay. The cells transfected with both HLA-B7 and MAGE-A1 stimulated CTL clone LB1803 483/G8.4 to produce TNF (**Figure 10B**). COS-7 cells transfected with HLA-B7 and either MAGE-A2,-A3,-A4,-A6,-

A8,-A9,-A10,-A11,-A12,-B1,-B2, or -C2 were unable to stimulate CTL clone LB1803 483/G8.4 to produce TNF.

Identification of the antigenic peptide

To identify the MAGE-A1 peptide recognized by CTL clone LB1803 483/G8.4, a set of peptides of 12 amino acids, that overlapped by 8 amino acids and covered the entire MAGE-A1 protein sequence, was screened. Autologous EBV-B cells were incubated with each of these peptides at a concentration of 2µg/ml, and tested for recognition by CTL clone LB1803 483/G8.4 in a chromium release assay at an effector to target cell ratio of 20:1. Peptide RVRFFFPSLREA (MAGE-A1 289-300) (SEQ ID NO: 56) scored positive. The sequence of this peptide was screened for an HLA-B7 binding peptide with the software available at "http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html". Peptide RVRFFFPSL (MAGE-A1 289-297) (SEQ ID NO: 57) had the highest score. It was tested in a cytotoxicity assay with CTL clone LB1803 483/G8.4 (E/T ratio of 20:1) and produced half-maximal lysis of autologous EBV-B target cells at ~ 20nM (**Figure 10D**).

Recognition by CTL clone LB1803 483/G8.4 of HLA-B7 cells expressing MAGE-A1.

CTL clone LB1803 483/G8.4 was also able to lyse HLA-B7 melanoma cell line ME275 clone 2 that expressed MAGE-A1 (**Figure 10D**). Melanoma cell line ME190DA was also lysed but only after treatment for 72h with 100 U/ml of IFN-gamma.

WE CLAIM:

1. A method for isolating, from a sample containing T cells of a subject, a cytotoxic T cell clone specific for a protein, comprising:

(a) contacting the sample with a first antigen presenting cell, wherein the expression of said protein in said first antigen presenting cell is effected by a first expression system;

(b) testing cells in said sample with a second antigen presenting cell and selecting for responding CTL cells, wherein the expression of said protein in said second antigen presenting cell is effected by a second expression system;

(c) contacting the cells selected in step (b) with a third antigen presenting cell, wherein the expression of said protein in said third antigen presenting cell is effected by a third expression system; and

(d) testing the cells in step (c) with a fourth antigen presenting cell and selecting for responding CTL cells, wherein the expression of said protein in said fourth antigen presenting cell is effected by a fourth expression system;

wherein each said antigen presenting cell is an autologous antigen presenting cell, and each of the expression systems is different from at least one of the

other expression systems, thereby isolating a cytotoxic T cell clone specific for said protein.

2. The method of claim 1, wherein said first, second, third or fourth expression system is selected from recombinant *Yersinia*, recombinant *Salmonella*, recombinant ALVAC, recombinant vaccinia, recombinant adenovirus, or recombinant retrovirus.

3. The method of claim 1, wherein each said antigen presenting cell is selected from the group consisting of a macrophage, dendritic cell, a B cell, a monocyte, marginal zone Kupffer cell, a Langerhans' cell, an interdigitating dendritic cell, a follicular dendritic cell, a T cell, an activated T cell, an astrocyte, a follicular cell, an endothelium cell and a fibroblast.

4. The method of claim 1, wherein said protein is selected from the group consisting of members of the MAGE family, the BAGE family, the DAGE/PRAME family, the GAGE family, the RAGE family, the SMAGE family, NAG, Tyrosinase, Melan-A/MART-1, gp100, MUC-1, TAG-72, CA125, p21ras, p53, HPV16 E7, the SSX family, HOM-MEL-55, NY-COL-2, HOM-HD-397, HOM-RCC-1.14, HOM-HD-21, HOM-NSCLC-11, HOM-MEL-2.4, HOM-TES-11, RCC-3.1.3, NY-ESO-1, and the SCP family.

5. The method of claim 1, wherein said testing is conducted by an assay selected from an IFN-gamma

secretion assay, a TNF production assay, a ^{51}Cr release assay, an ELI-spot assay or a tetramer staining assay.

6. A method for identifying an antigenic peptide epitope of a protein, comprising:

(a) obtaining a sample containing T cells and contacting the sample with a first antigen presenting cell, wherein the expression of said protein in said first antigen presenting cell is effected by a first expression system;

(b) testing cells in said sample with a second antigen presenting cell and selecting for responding CTL cells, wherein the expression of said protein in said second antigen presenting cell is effected by a second expression system;

(c) contacting the cells selected in step (b) with a third antigen presenting cell, wherein the expression of said protein in said third antigen presenting cell is effected by a third expression system; and

(d) testing the cells in step (c) with a fourth antigen presenting cell and selecting for responding CTL cells, wherein the expression of said protein in said fourth antigen presenting cell is effected by a fourth expression system;

wherein the antigen presenting cells are immunocompatible with each other, and each of the expression systems is different from at least one of the other expression systems, thereby isolating a cytotoxic T cell clone specific for said protein; and

(e) identifying an antigenic peptide epitope of said protein by using the cytotoxic T cell clone isolated in step (d).

7. A CTL clone isolated by the method of claim 1.

8. An isolated CTL clone specific for a peptide/HLA complex, wherein said complex is selected from the group consisting of SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48)/HLA-A28, AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and RVRFFFPSL (SEQ ID NO: 57)/HLA-B7.

9. An isolated CTL clone, wherein said clone is selected from the group consisting of LB1137 462/F3.2, LB1801 456/H7.11, LB1137 H4.13, LB1118 466/D3.31, LB1801 456/H8.33, LB1841 526/F7.1 and LB1803 483/G8.4.

10. An isolated antigenic peptide, wherein said peptide is selected from the group consisting of DPARYEFLW(SEQ ID NO: 42), GVYDGREHTV(SEQ ID NO: 44), SAFPTTINF(SEQ ID NO: 47), EVYDGREHSA(SEQ ID NO: 48), AELVHFLLL (SEQ ID NO: 55) and RVRFFFPSL (SEQ ID NO: 57).

11. An isolated nucleic acid molecule encoding said peptide of claim 10.

12. An isolated peptide/HLA complex, wherein said complex is selected from the group consisting of SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48)/HLA-A28, AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and RVRFFFPSL (SEQ ID NO: 57)/HLA-B7.

13. An antigen presenting cell expressing said peptide/HLA complex of claim 12.

14. A pharmaceutical composition comprising a pharmaceutical carrier and said CTL clone of claim 7.

15. A pharmaceutical composition comprising a pharmaceutical carrier and at least one isolated CTL clone selected from the group consisting of a CTL clone specific for SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, a CTL clone specific for DPARYEFLW(SEQ ID NO: 42)/HLA-B53, a CTL clone specific for GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, a CTL clone specific for SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, a CTL clone specific for EVYDGREHSA(SEQ ID NO: 48)/HLA-A28, a CTL clone specific for AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and a CTL clone specific for RVRFFFPSL (SEQ ID NO: 57)/HLA-B7.

16. A pharmaceutical composition comprising a pharmaceutical carrier and at least one peptide selected from the group consisting of DPARYEFLW(SEQ ID NO: 42),

GVYDGREHTV(SEQ ID NO: 44), SAFPTTINF(SEQ ID NO: 47), EVYDGREHSA(SEQ ID NO: 48), AELVHFLLL (SEQ ID NO: 55) and RVRFFFPSL (SEQ ID NO: 57).

17. A pharmaceutical composition comprising a pharmaceutical carrier and at least one isolated peptide/HLA complex selected from the group consisting of SAYGEPRKL (SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48)/HLA-A28, AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and RVRFFFPSL (SEQ ID NO: 57)/HLA-B7.

18. A pharmaceutical composition comprising a pharmaceutical carrier and at least one antigen presenting cell selected from the group consisting of an antigen presenting cell expressing SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, an antigen presenting cell expressing DPARYEFLW(SEQ ID NO: 42)/HLA-B53, an antigen presenting cell expressing GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, an antigen presenting cell expressing SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, an antigen presenting cell expressing EVYDGREHSA(SEQ ID NO: 48)/HLA-A28, an antigen presenting cell expressing AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and an antigen presenting cell expressing RVRFFFPSL (SEQ ID NO: 57)/HLA-B7.

19. A method of detecting in a sample from a subject, the presence of a cell which abnormally expresses a peptide/HLA complex at the cell surface,

wherein said peptide/HLA complex is any one of SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48)/HLA-A28, AELVHFLLL(SEQ ID NO: 55)/HLA-B40, or RVRFFFPSL(SEQ ID NO: 57)/HLA-B7, said method comprising contacting said sample with a CTL clone specific for said complex.

20. A method of diagnosing in a subject, a pathological condition characterized by an abnormal expression of a peptide/HLA complex, wherein said peptide/HLA complex is selected from the group consisting of SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48)/HLA-A28, AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and RVRFFFPSL(SEQ ID NO: 57)/HLA-B7, said method comprising detecting in the subject, the presence of cells which abnormally express said peptide/HLA complex at the cell surface.

21. A method of detecting in a sample of a subject, the presence of CTL cells specific for a peptide/HLA complex, wherein said complex is selected from the group consisting of SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48)/HLA-A28, AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and RVRFFFPSL(SEQ ID NO: 57)/HLA-B7, said method comprising contacting said sample with a cell

which presents said peptide/HLA complex on the surface of said cell, and detecting a CTL response as an indication of the presence of said CTL cells.

22. A method of diagnosing in a subject, a pathological condition characterized by an abnormal expression of a peptide/HLA complex, wherein said peptide/HLA complex is selected from the group consisting of SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48)/HLA-A28,_AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and RVRFFFPSL(SEQ ID NO: 57)/HLA-B7, said method comprising detecting in the subject, an increased frequency of CTL cells specific for said complex.

23. A method of treating a subject having a pathological condition characterized by abnormal expression of a peptide/HLA complex, wherein said complex is selected from the group consisting of SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48)/HLA-A28, AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and RVRFFFPSL(SEQ ID NO: 57)/HLA-B7, comprising administering to said subject, a therapeutically effective amount of CTL cells specific for said complex.

24. A method of treating a subject having a pathological condition characterized by abnormal

expression of a peptide/HLA complex, wherein said complex is selected from the group consisting of SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48)/HLA-A28, AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and RVRFFFPSL(SEQ ID NO: 57)/HLA-B7, comprising administering to said subject, a therapeutically effective amount of the peptide in said peptide/HLA complex.

25. A method of treating a subject having a pathological condition characterized by abnormal expression of a peptide/HLA complex, wherein said complex is selected from the group consisting of SAYGEPRKL(SEQ ID NO: 2)/HLA-Cw3, DPARYEFLW(SEQ ID NO: 42)/HLA-B53, GVYDGREHTV(SEQ ID NO: 44)/HLA-A2, SAFPTTINF(SEQ ID NO: 47)/HLA-Cw2, EVYDGREHSA(SEQ ID NO: 48)/HLA-A28, AELVHFLLL(SEQ ID NO: 55)/HLA-B40, and RVRFFFPSL(SEQ ID NO: 57)/HLA-B7, comprising administering to said subject, a therapeutically effective amount of cells which present said complex on the cell surface.

26. The method of claim 25, wherein said cells are autologous antigen presenting cells and wherein the presentation of said said peptide/HLA complex on the cell surface is achieved by loading said cells with the peptide in said peptide/HLA complex.

27. The method of claim 25, wherein said cells are autologous antigen presenting cells and wherein the

presentation of said peptide/HLA complex on the cell surface is achieved by transfecting said cells with a nucleotide sequence coding for the peptide in said peptide/HLA complex.

FIG. 1

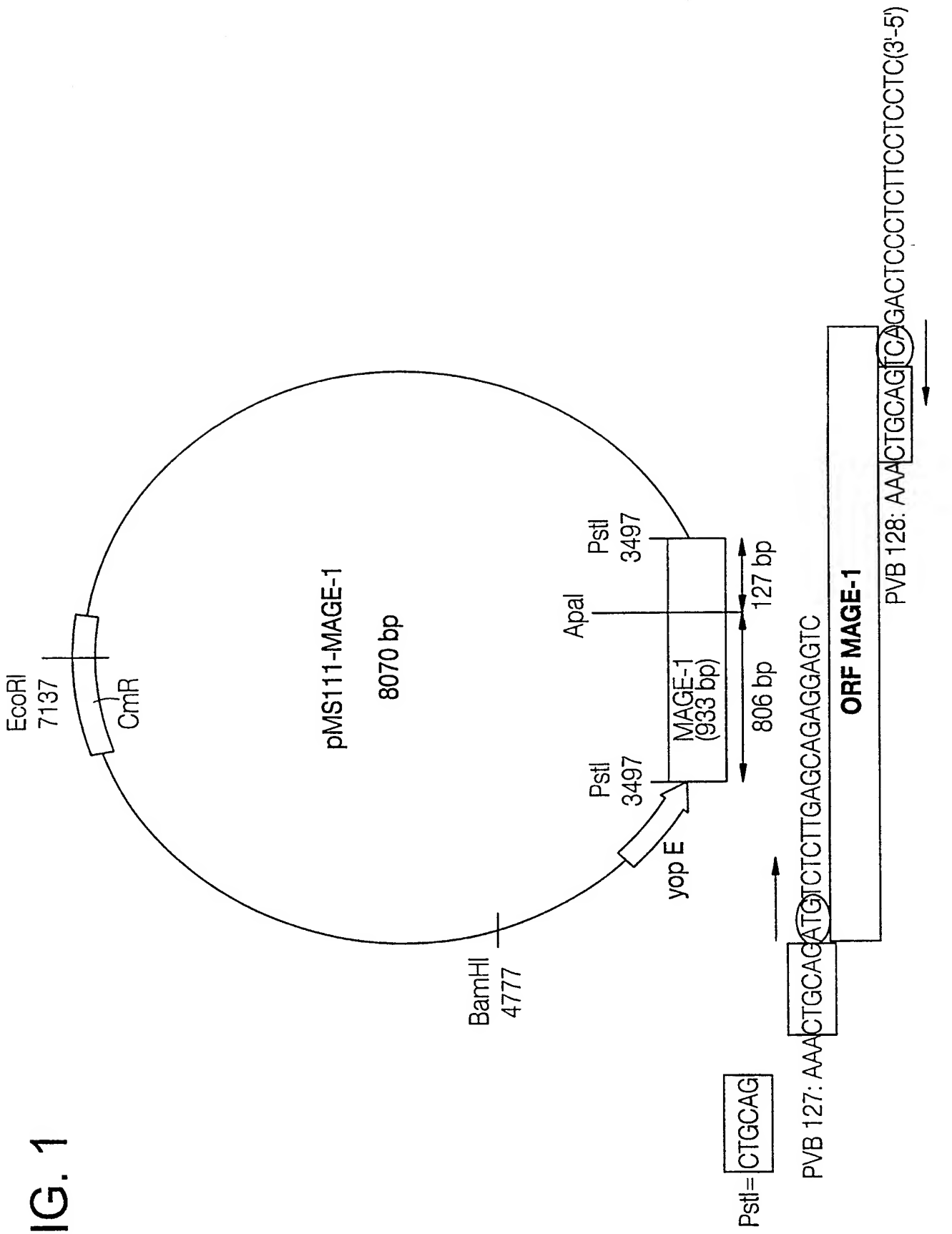

EcoRI
7137
CmR
pMS111-MAGE-1
8070 bp
BamHI
4777
yop E
PstI
3497
MAGE-1
(933 bp)
ApaI
PstI
3497
806 bp
127 bp
PstI= CTGCAG
PVB 127: AAACTGCAGATGTCTCTTGAGCAGAGGAGTC
ORF MAGE-1
PVB 128: AAACTGCAGTCAGACTCCCTCTTCCTCCTC(3'-5')

FIG. 2A

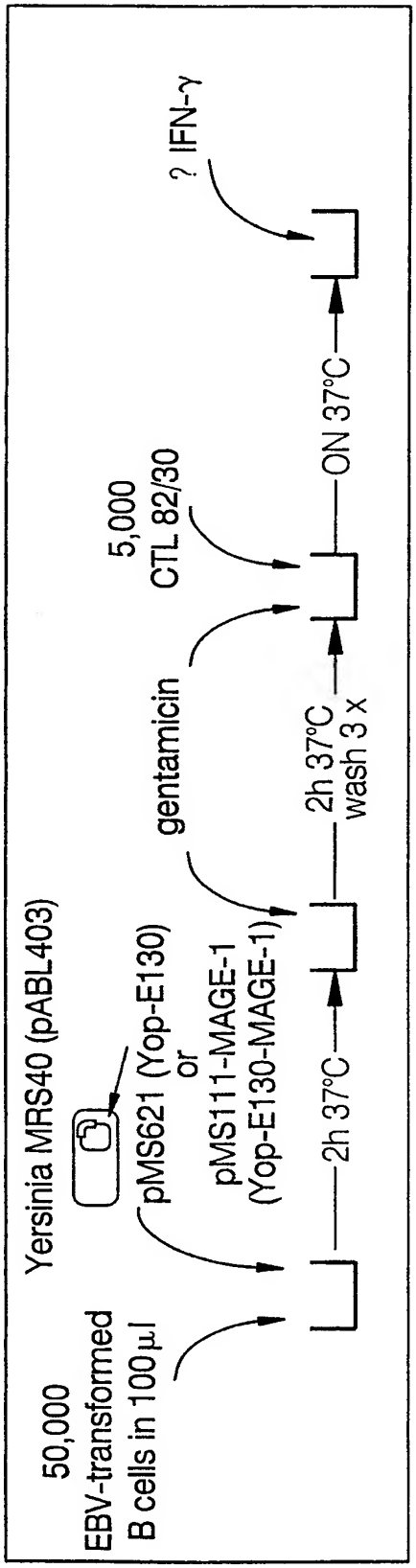
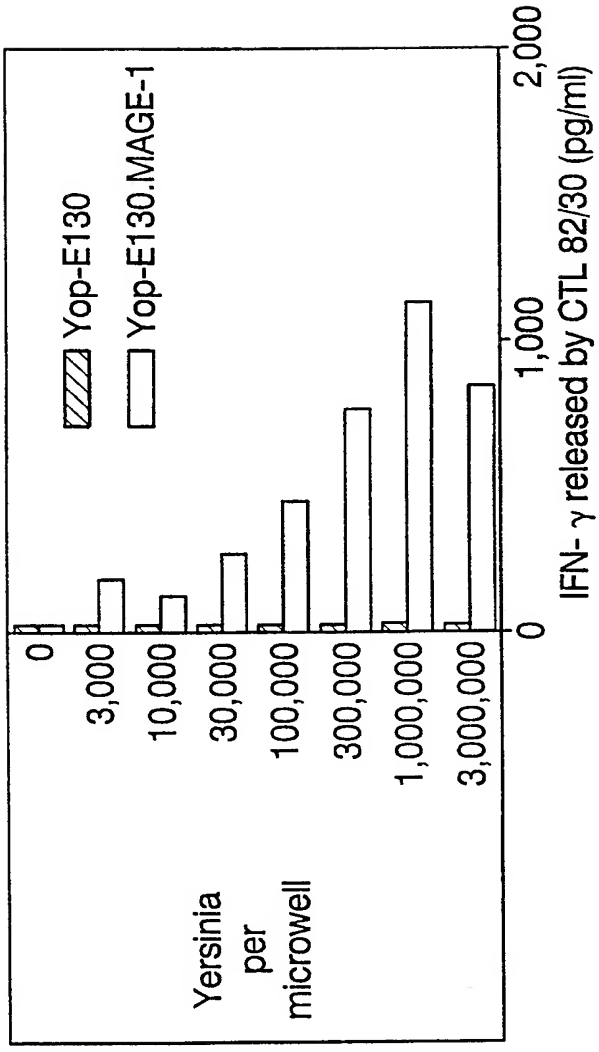

50,000
EBV-transformed
B cells in 100µl
Yersinia MRS40 (pABL403)
pMS621 (Yop-E130)
or
pMS111-MAGE-1
(Yop-E130-MAGE-1)
2h 37°C
gentamicin
2h 37°C
wash 3 x
5,000
CTL 82/30
ON 37°C
? IFN-γ

FIG. 2B


Yop-E130
Yop-E130.MAGE-1
Yersinia per microwell
0
3,000
10,000
30,000
100,000
300,000
1,000,000
3,000,000
0
1,000
2,000
IFN- γ released by CTL 82/30 (pg/ml)

A MAGE-1 antigenic peptide is presented by HLA-Cw3 to CTL clone F3/2

FIGURE 3A

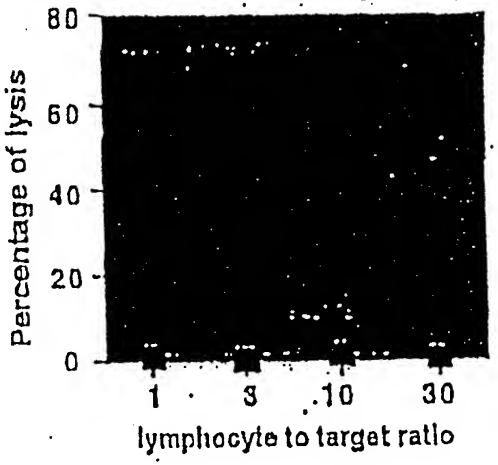
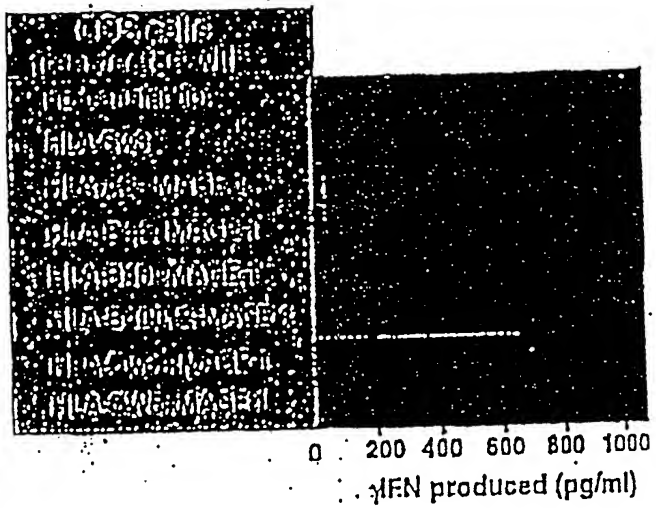


FIGURE 3B



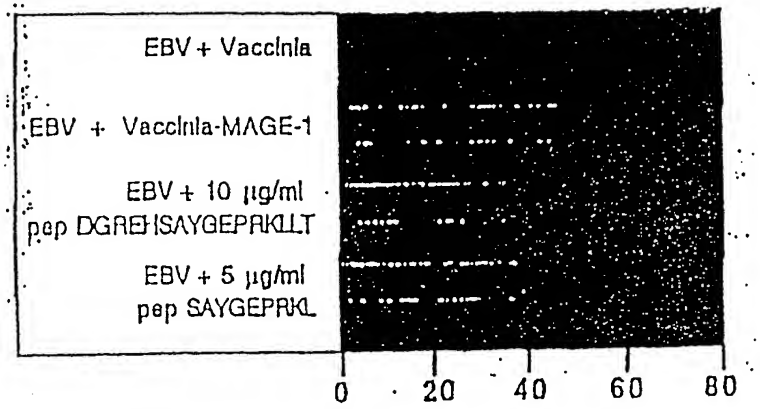


FIGURE 3C

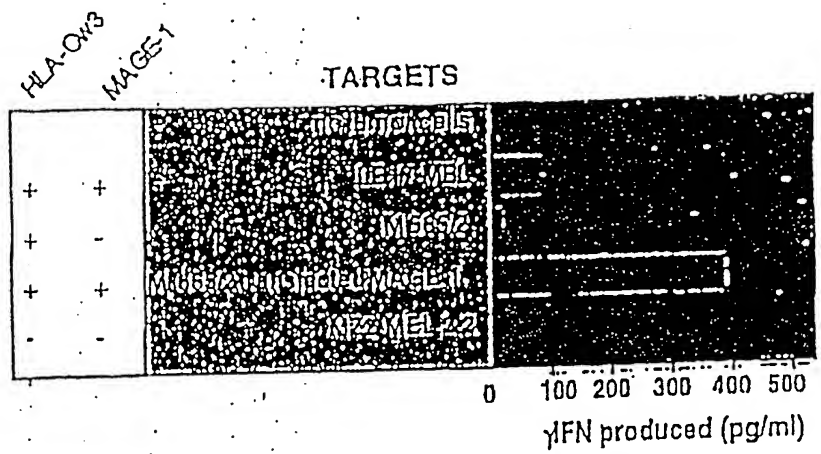


FIGURE 3D

FIGURE 3

1998-PvdB-22 (M1Cw3) CTL)

A MAGE-1 antigenic peptide is presented by HLA-B53 to CTL clone 456/H7.11

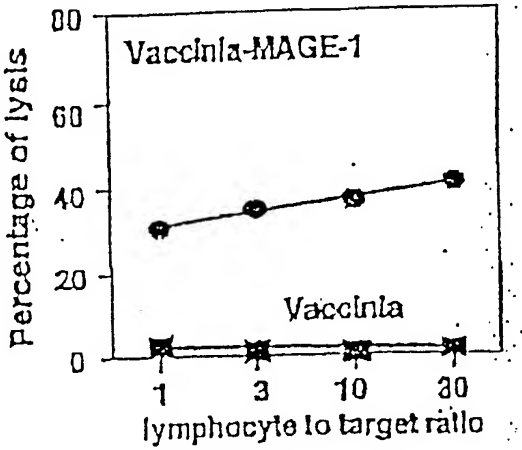
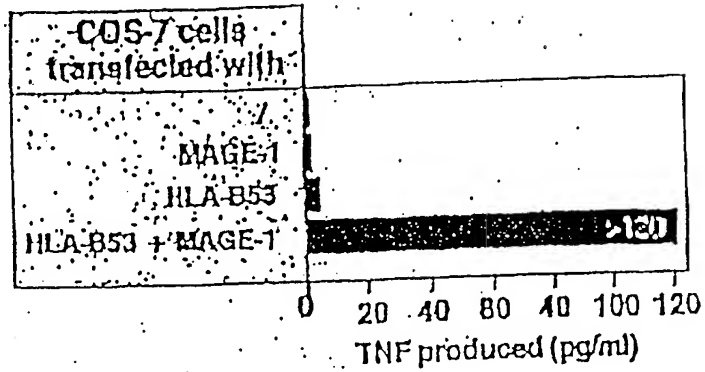


FIGURE 4A

FIGURE 4B



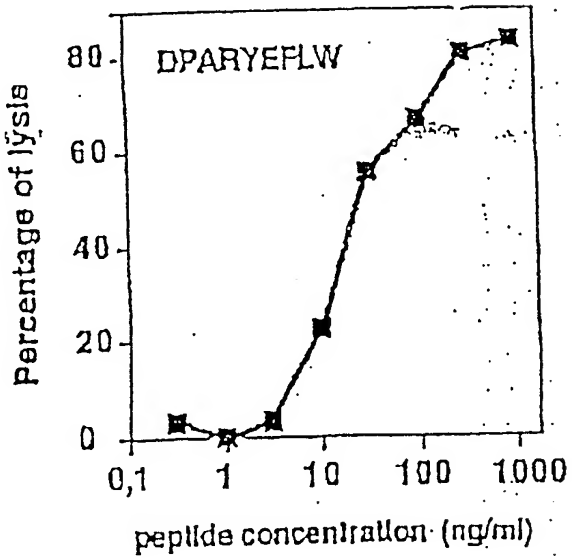


FIGURE 4C

No tumor cell line available
MAGE-1 +
HLA-B53 +

FIGURE 4

Figure 5

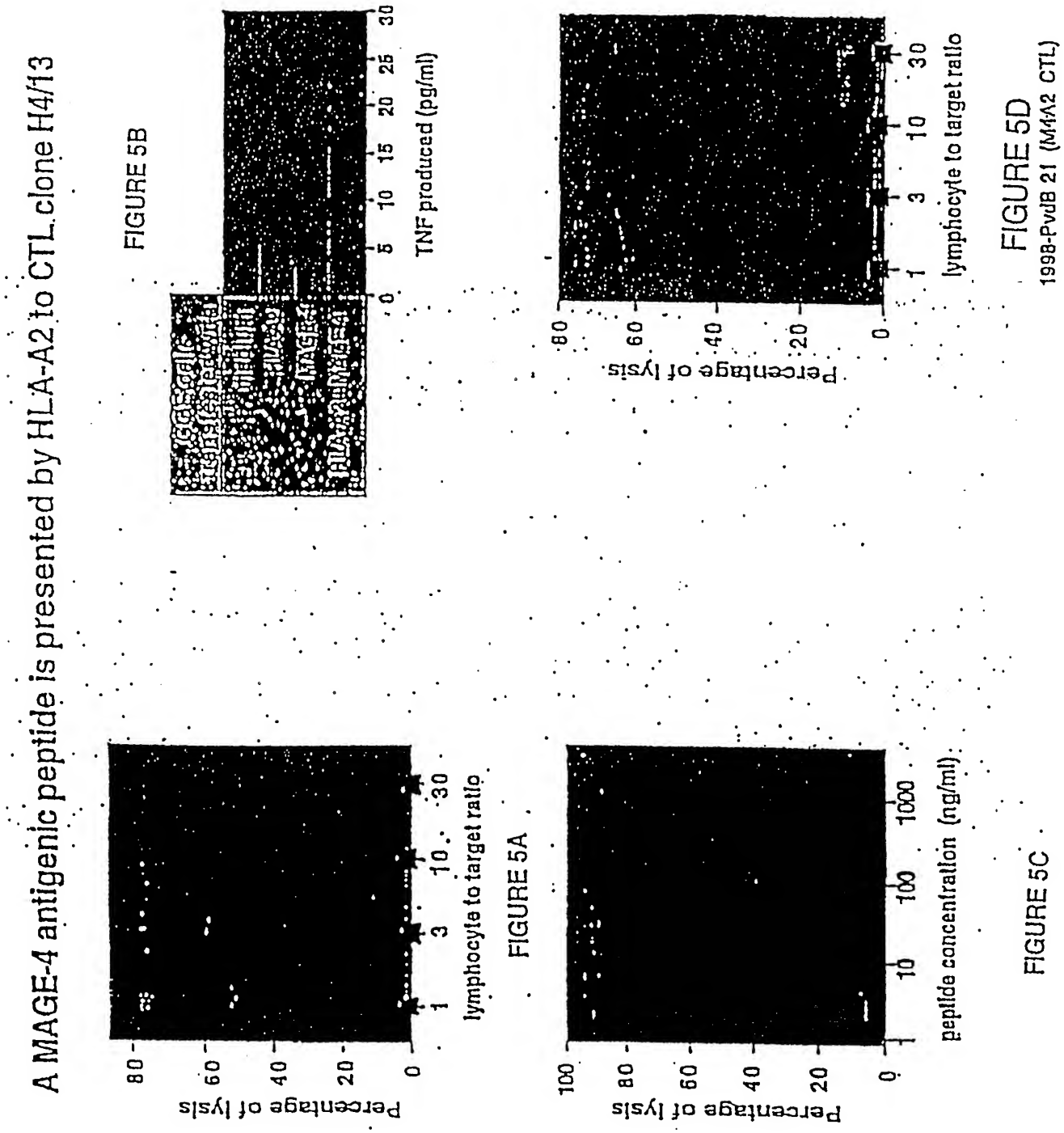

A MAGE-4 antigenic peptide is presented by HLA-A2 to CTL clone H4/13
FIGURE 5A
Percentage of lysis
lymphocyte to target ratio
FIGURE 5B
TNF produced (pg/ml)
FIGURE 5C
Percentage of lysis
peptide concentration (ng/ml)
FIGURE 5D
1998-PvdB 21 (MAA2 CTL)
Percentage of lysis
lymphocyte to target ratio

FIG. 6

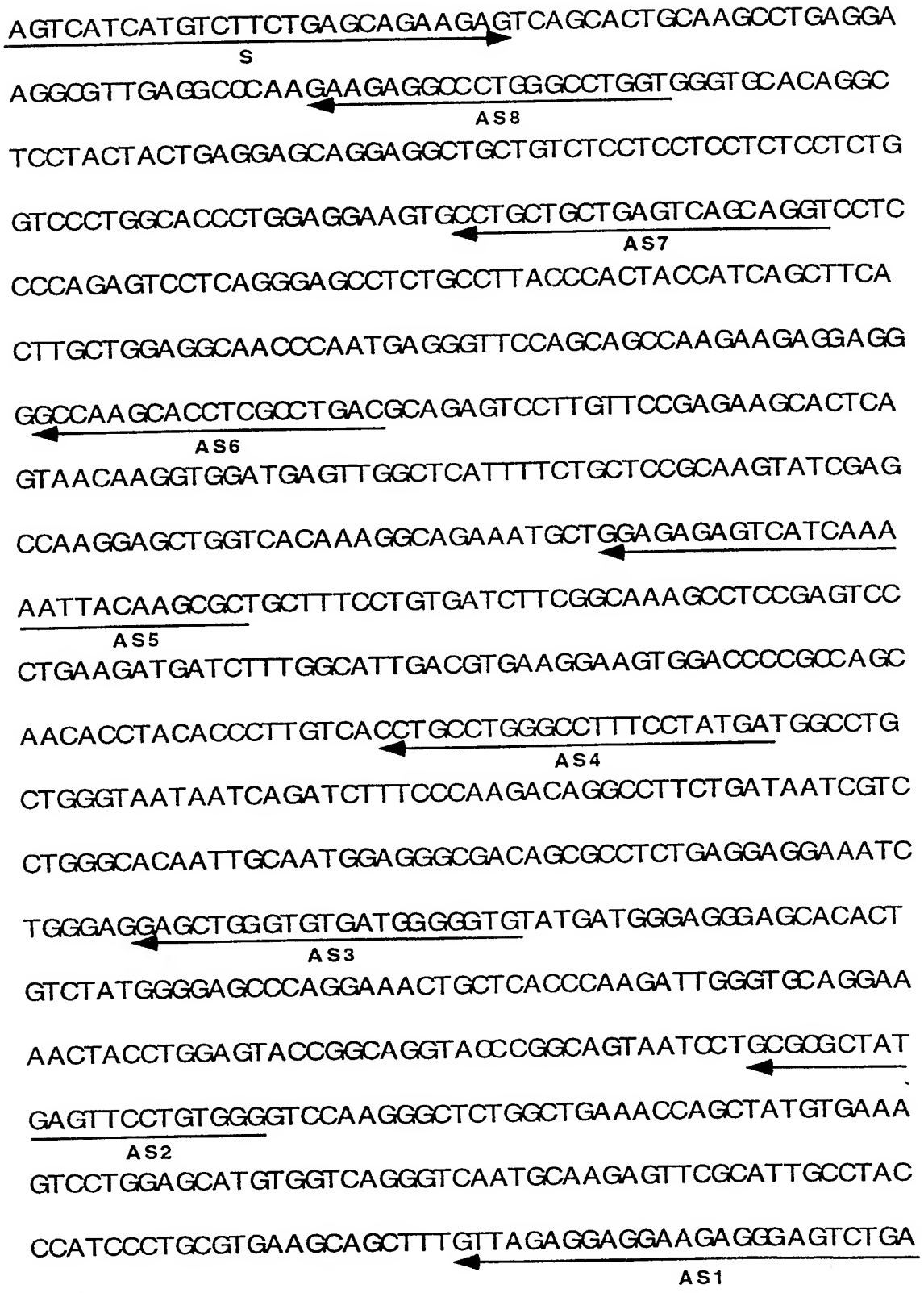

AGTCATCATGTCTTCTGAGCAGAAGAGTCAGCACTGCAAGCCTGAGGA
S
AGGCGTTGAGGCCCAAGAAGAGGCCCTGGGCCTGGTGGGTGCACAGGC
AS8
TCCTACTACTGAGGAGCAGGAGGCTGCTGTCTCCTCCTCCTCTCCTCTG
GTCCCTGGCACCCTGGAGGAAGTGCCTGCTGCTGAGTCAGCAGGTCCTC
AS7
CCCAGAGTCCTCAGGGAGCCTCTGCCTTACCCACTACCATCAGCTTCA
CTTGCTGGAGGCAACCCAATGAGGGTTCCAGCAGCCAAGAAGAGGAGG
GGCCAAGCACCTCGCCTGACGCAGAGTCCTTGTTCCGAGAAGCACTCA
AS6
GTAACAAGGTGGATGAGTTGGCTCATTTTCTGCTCCGCAAGTATCGAG
CCAAGGAGCTGGTCACAAAGGCAGAAATGCTGGAGAGAGTCATCAAA
AATTACAAGCGCTGCTTTCCTGTGATCTTCGGCAAAGCCTCCGAGTCC
AS5
CTGAAGATGATCTTTGGCATTGACGTGAAGGAAGTGGACCCCGCCAGC
AACACCTACACCCTTGTCACCTGCCTGGGCCTTTCCTATGATGGCCTG
AS4
CTGGGTAATAATCAGATCTTTCCCAAGACAGGCCTTCTGATAATCGTC
CTGGGCACAATTGCAATGGAGGGCGACAGCGCCTCTGAGGAGGAAATC
TGGGAGGAGCTGGTGTGATGGGGGTGTATGATGGGAGGGAGCACACT
AS3
GTCTATGGGGAGCCCAGGAAACTGCTCACCCAAGATTGGGTGCAGGAA
AACTACCTGGAGTACCGGCAGGTACCCGGCAGTAATCCTGCGCGCTAT
GAGTTCCTGTGGGGTCCAAGGGCTCTGGCTGAAACCAGCTATGTGAAA
AS2
GTCCTGGAGCATGTGGTCAGGGTCAATGCAAGAGTTCGCATTGCCTAC
CCATCCCTGCGTGAAGCAGCTTTGTTAGAGGAGGAAGAGGGAGTCTGA
AS1

FIG. 7A

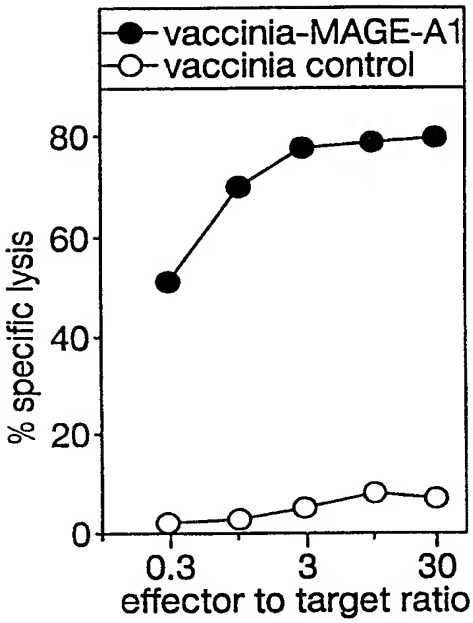


FIG. 7B

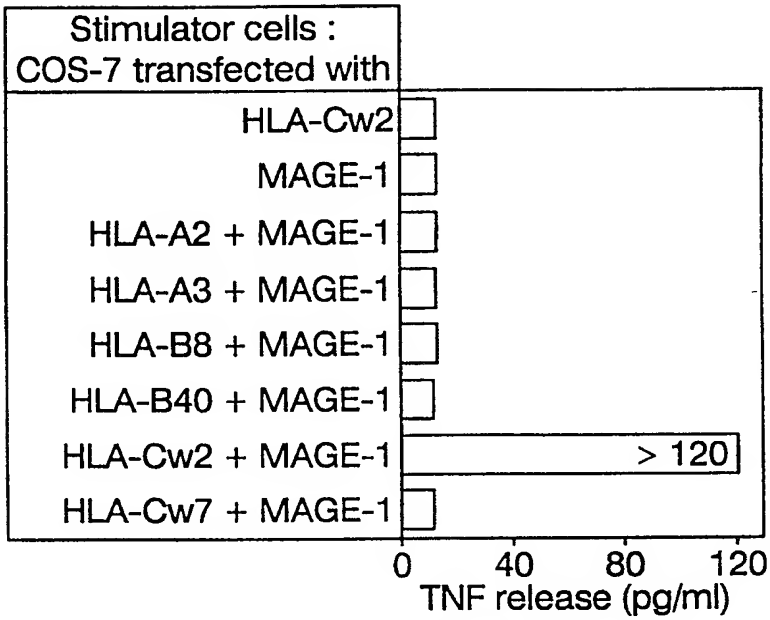


FIG. 7C

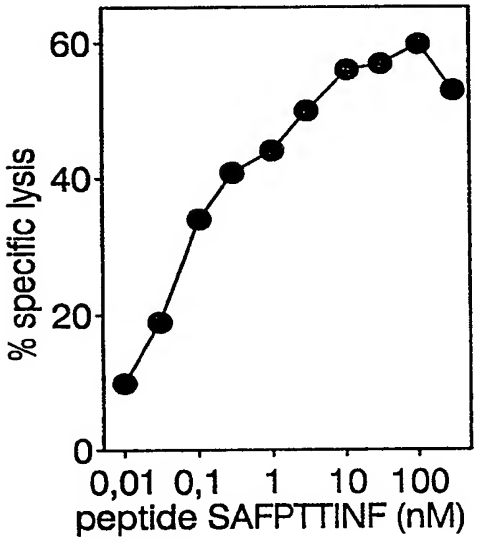


FIG. 7D

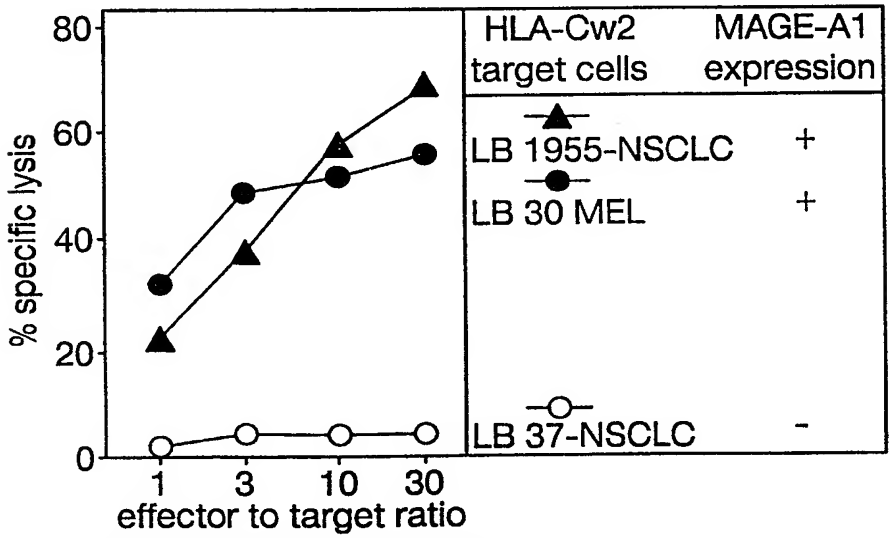


FIG. 8A

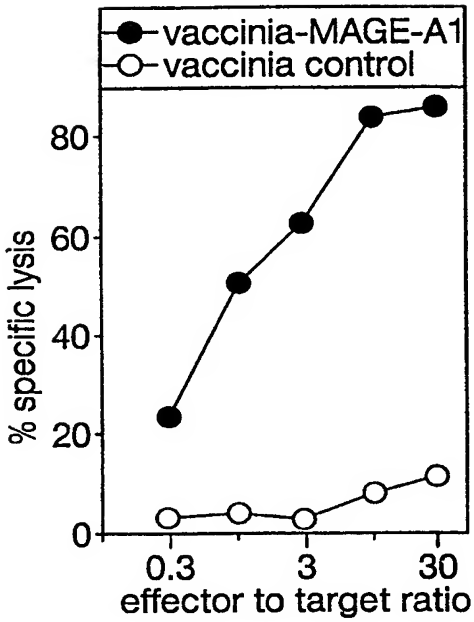


FIG. 8B

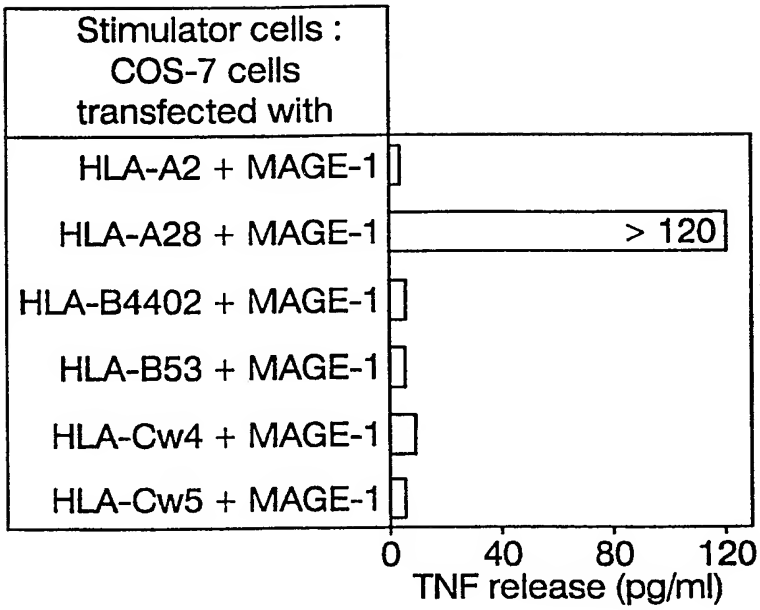


FIG. 8C

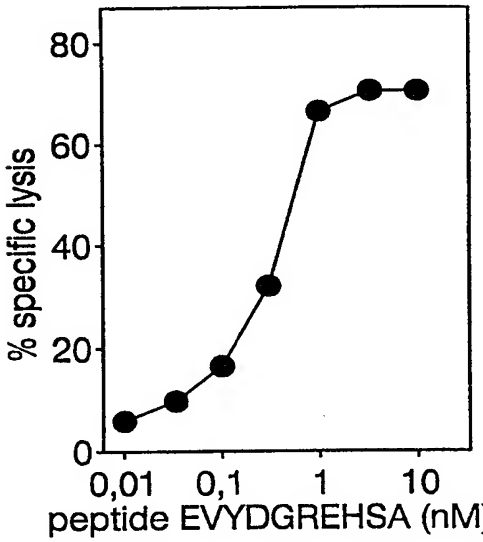


FIG. 8D

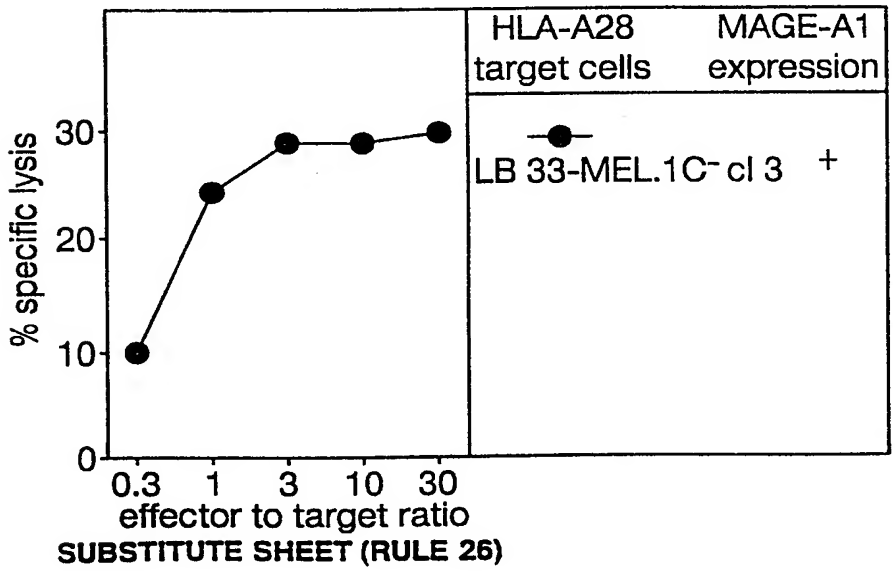


FIG.9A

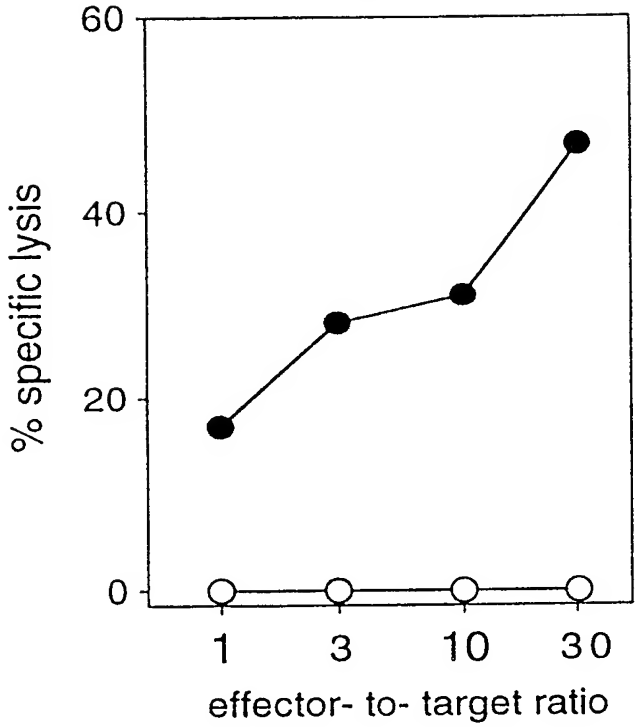

60
40
20
0
% specific lysis
1
3
10
30
effector- to- target ratio

FIG.9B

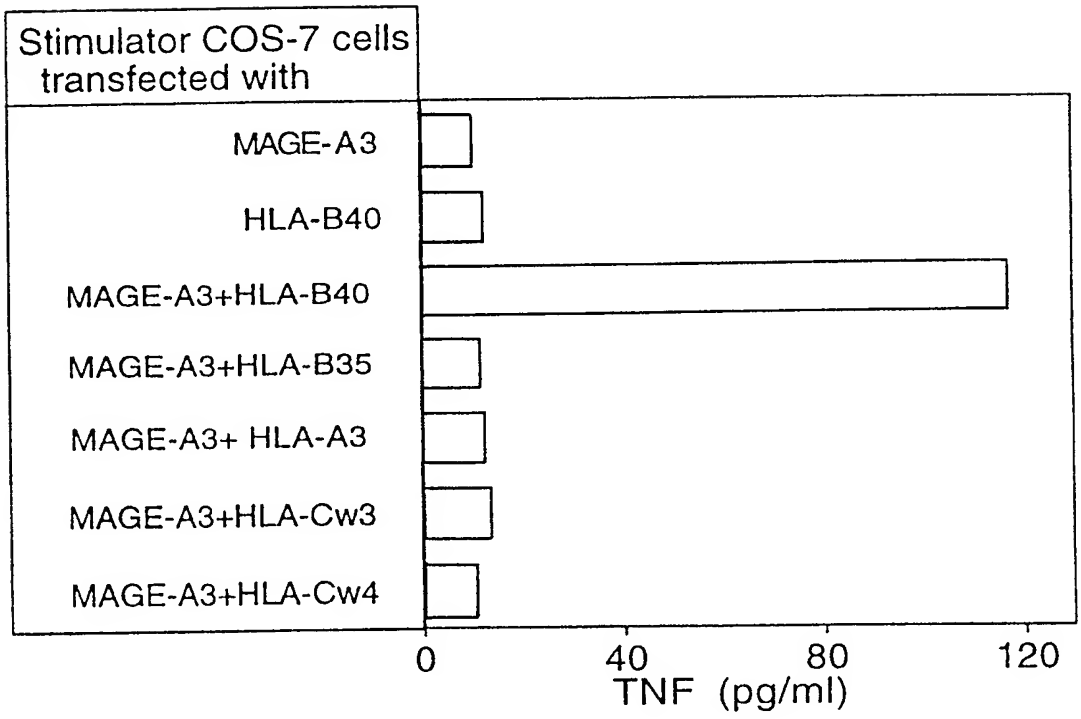

Stimulator COS-7 cells transfected with
MAGE-A3
HLA-B40
MAGE-A3+HLA-B40
MAGE-A3+HLA-B35
MAGE-A3+ HLA-A3
MAGE-A3+HLA-Cw3
MAGE-A3+HLA-Cw4
0
40
80
120
TNF (pg/ml)

FIG.9C

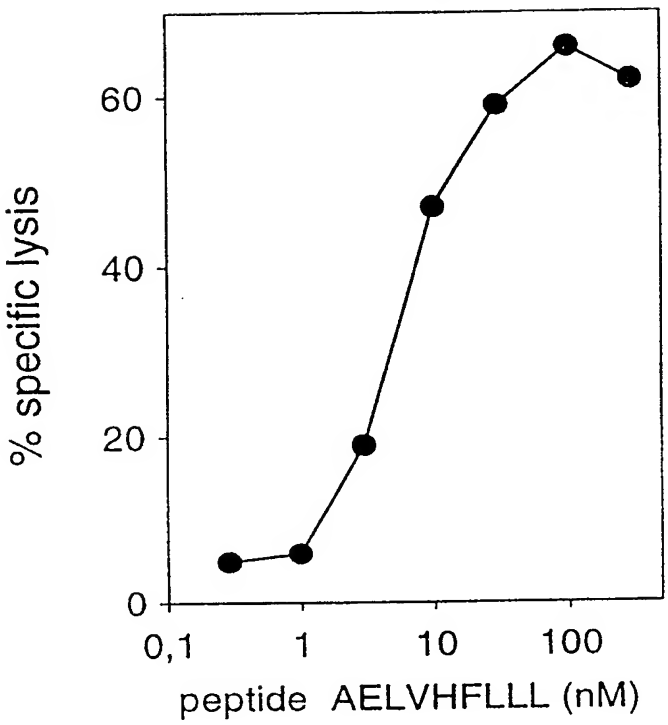

% specific lysis
60
40
20
0
0,1
1
10
100
peptide AELVHFLLL (nM)

FIG.9D

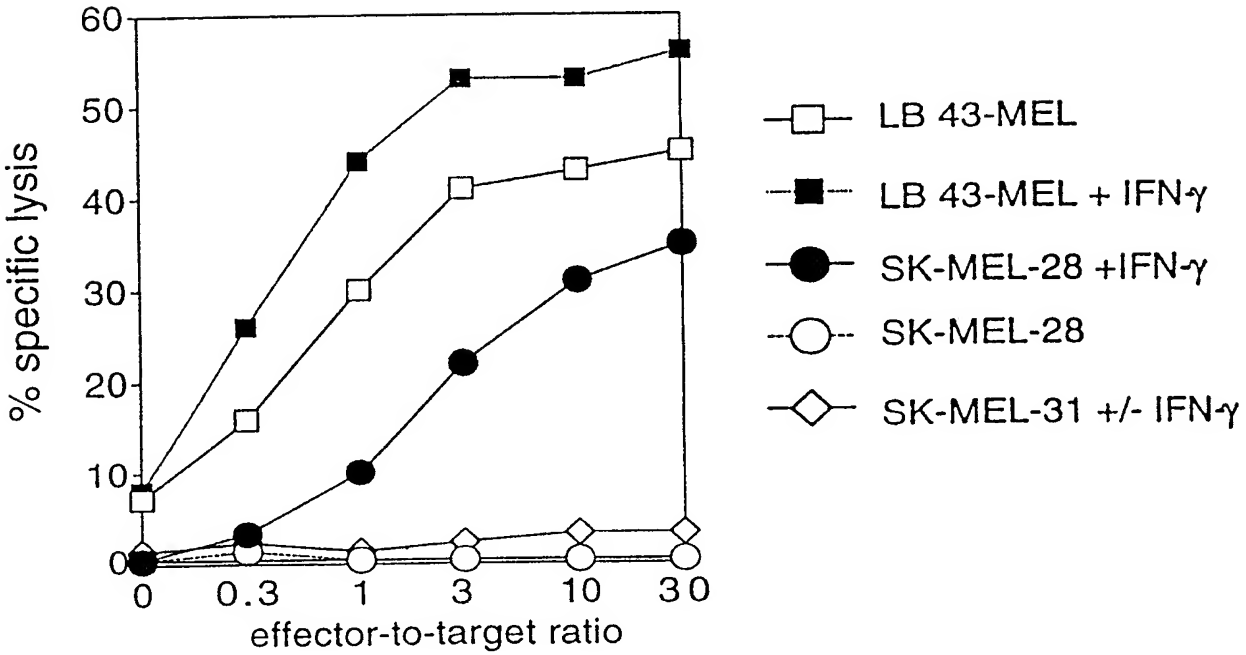

% specific lysis
60
50
40
30
20
10
0
0
0.3
1
3
10
30
effector-to-target ratio
LB 43-MEL
LB 43-MEL + IFN-γ
SK-MEL-28 +IFN-γ
SK-MEL-28
SK-MEL-31 +/- IFN-γ

FIG.10A

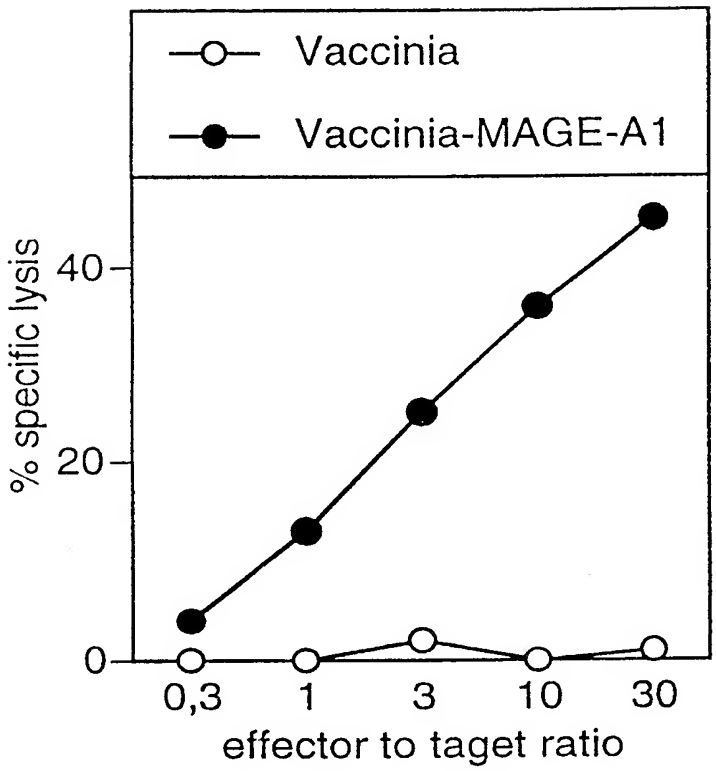


FIG.10B

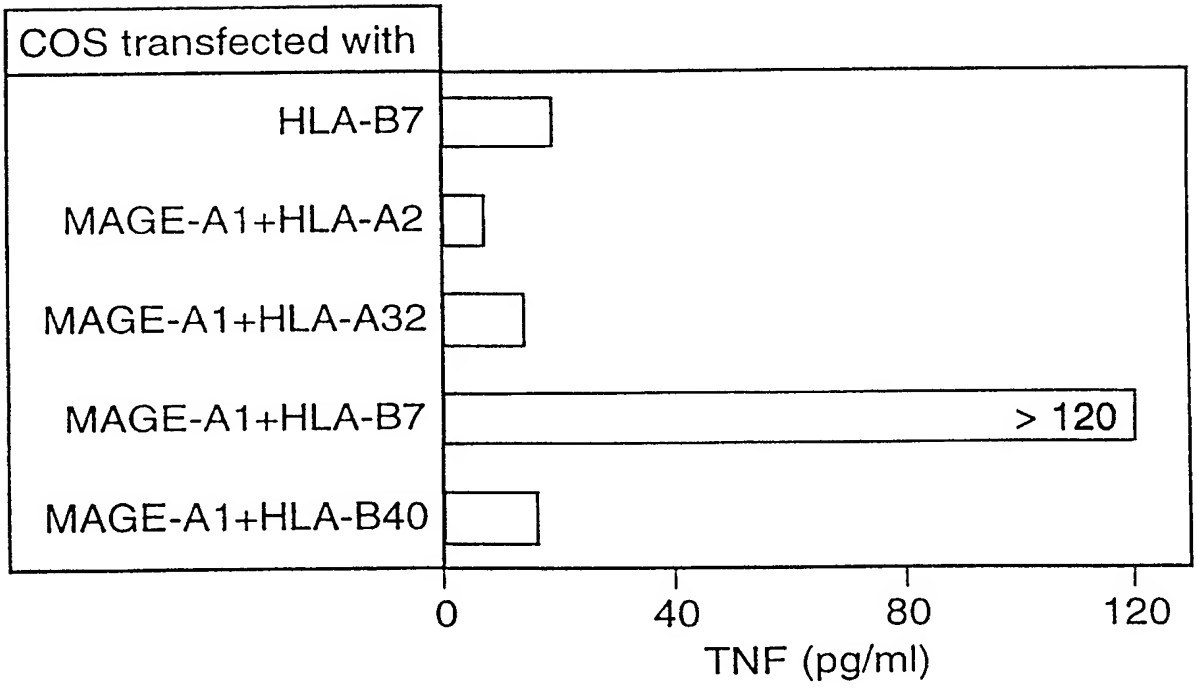


FIG.10C

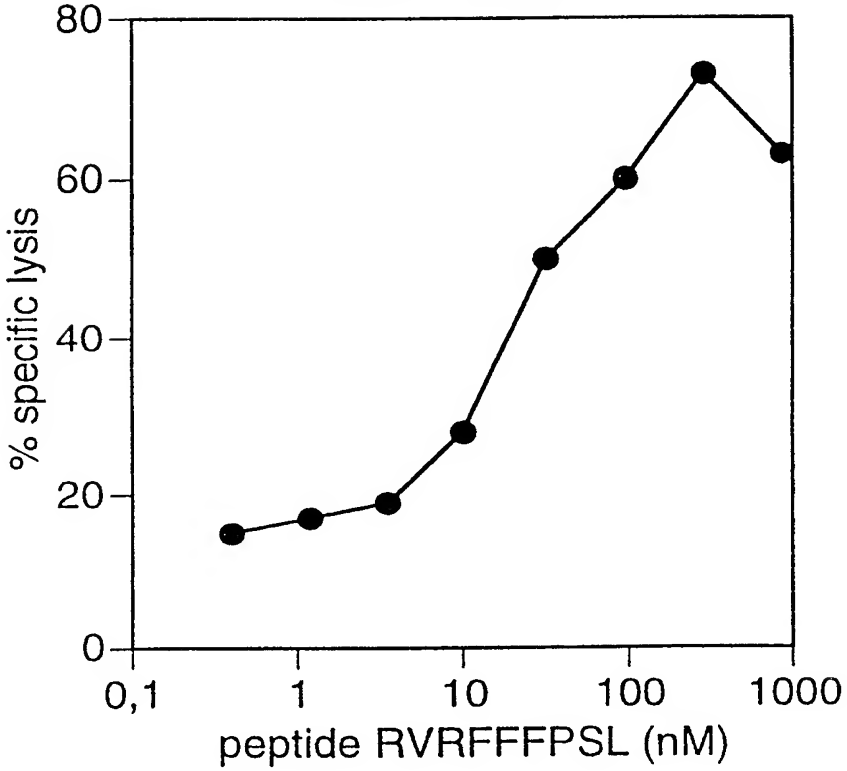


FIG.10D